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On the possible iodometric estimation of insulin.

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It is generally agreed that insulin is destroyed by trypsin and other proteolytic enzymes. Recently,[1] we suggested that the physiologically inactive addition compound, which is formed between insulin and trypsin before the digestion of insulin starts, is probably the so-called intermediate compound between enzyme and substrate. Up to the present, conclusions as to the existence or non-existence of the enzyme-substrate compound have been drawn almost exclusively[2] from the analysis of kinetic studies. Further progress in the investigation of the insulin-enzyme reaction depends in part on the development of a suitable method for the chemical estimation of insulin.

Certain earlier observations[3] induced us to attempt an iodome-

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[1] Brand, E., and Sandberg, M., PROC. SOC. EXP. BIOL. AND MED., 1925, xxii, 428.

[2] *Cf.* Bergmann, M., *Liebigs Ann.*, 1925, cdliii, 223.

[3] In 1922, one of us (B.) carried out with Dr. Benno Brahn some experiments on the iodometric estimation of epinephrin in acid solution. We shall report on these studies in the near future.

tric estimation of insulin. Our present knowledge of the chemical constitution of insulin is not opposed to the feasibility of such a method. We know from studies on the iodization of proteins[4] that iodine reacts with various groups of the protein molecule, among others with the lead-blackening sulphur. The presence of lead-blackening sulphur in the molecule of insulin seems to have been demonstrated by Abel and Geiling.[5] Whether the insulin molecule contains other groups that react with iodine cannot yet be decided. It has been recommended that the iodometric estimation of certain inorganic groups be carried out in the presence of neutral buffer. We have noticed that phenols, glucose, cyclic aminoacids, and other substances, which are supposed to react with I_2-KI solution only at an alkaline pH, take up iodine also in the presence of neutral buffer.[6]

For the iodization and iodometric estimation of organic compounds the use of neutral-buffered I_2-KI solution offers an additional method, which may be of considerable value both preparatively and analytically. It will also be interesting to study bromine oxidations by using bromine-neutral buffer instead of bromine-alkali or bromine-alkali carbonate.

In acid solution, insulin (20 units per mg.) immediately forms a reddish brown voluminous precipitate with I_2-KI solution (formation of a periodide?). In neutral-buffered or alkaline solution no precipitation takes place at first. After standing for some time and with slight heating a yellow precipitate appears. After iodization in neutral-buffered or alkaline solution the test for lead-blackening sulphur is no longer positive.

An iodometric estimation of insulin can be attempted successfully only with material of a certain degree of purity. At lower stages of purification, groups that are present, though not belonging to insulin proper, will also absorb iodine. The majority of the preparations we used seem to contain approximately 15 to 20 units of insulin per mg. The iodine value of an older preparation containing only 8 units per mg., however, is irregular and far too high.

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[4] *Cf.* Blum, F., and Strauss, E., *Z. physiol. Chem.*, 1921, cxii, 111.

[5] Abel, J. J., and Geiling, E. M. K., *J. Pharmacol. and Exp. Therap.*, 1925, xxv, 422. *Cf.* Taylor, T. C., Braun, C. E., and Scott, E. L., *Am. J. Physiol.*, 1925, lxxiv, 539.

[6] We shall report on iodization and iodometric estimation of organic compounds in the presence of neutral buffer in another publication.

The method consists of permitting an excess of iodine (20 cc. of 0.01 N iodine solution) to react with insulin (twice precipitated at the isoelectric point in order to remove the preservative) in the presence of a neutral buffer (M/5 phosphate buffer pH=6.8) under standard conditions (17 hours, 37° C.), and estimating by titration (in 0.1 normal acid solution) the amount of iodine used. We find that the amount of iodine consumed by the insulin is directly proportional to the number of units present. Different batches of insulin from different manufacturers require approximately the same amount of iodine for an equal number of units. The iodine values of different preparations of insulin agree, as closely as can be expected, from material which has been standardized physiologically. Forty clinical units of insulin use 2.05 (±0.05) cc. of 0.01 N iodine solution, but this cannot yet be considered as the final value.

The iodine value of insulin at an alkaline reaction is higher than in neutral-buffered solution. But the alkaline iodine value

Insulin Preparation	cc. 0.01 N iodine.					
	pH = 6.8 (3 cc. M/5 phosphate buffer)			alkaline (1 cc. 2N NaOH)		
	20 units	40 units	60 units	20 units	40 units	60 units
Squibb U 20 Lot 29376	1.15	2.30	3.35	1.80	3.20	—
Lilly U 20 Lot 774002 16.7 units per mg.	1.02	2.00	3.00	1.64	2.90	4.01
Lilly U 40 Lot R-511	1.10	2.10	—	1.60	2.80	—
Lilly U 440 Lot 769999 20 units per mg.	(22 un.) 1.11	(44 un.) 2.17	(66 un.) 3.18	(22 un.) 1.75	(44 un.) 3.45	(66 un.) 5.12
Toronto U 40 Special Nov. 3, 1925.	1.03	2.12	3.12	2.37	4.20	5.90
Toronto U 20 Lot 318-25	1.06	2.12	3.17	1.80	3.15	4.25
Toronto U 10 Lot 317-48	1.07	2.08	—	1.70	2.98	—
Lilly—powdered Lot 441146 8 units per mg.	1.78	3.51	5.43	2.72	4.87	6.65

is not as nearly proportional to the unitage as in neutral-buffered solution. Moreover, preparations of insulin with the same neutral values have different alkaline iodine values.

The higher alkaline iodine value of insulin may be due to different causes:

(1) Hydrolysis may change the insulin preparation in such a way that more iodine can react. Our experiments show, however, that after hydrolysis with 2N NaOH or 2N HCl (48 hours 37°C.) the original neutral and alkaline iodine values are but slightly changed.

(2) Certain groups may be present that react with iodine at only an alkaline reaction. These groups may belong either to the insulin molecule itself or to impurities. It is, therefore, possible that pure insulin preparations will show the same iodine value in neutral and alkaline solution.

(3) An alkaline iodine solution may oxidize the same groups more strongly than does a neutral-buffered iodine solution.

It cannot yet be decided which of the suggestions offered under (2) and (3) is correct.

Our results seem to indicate that if the insulin preparations are of sufficient purity the number of units can be established iodometrically, using a neutral-buffered I_2-KI solution. The value of chemical estimations of physiologically active substances has been sufficiently discussed in the case of epinephrin.[7]

It seems possible that the iodometric estimation of insulin will be of some value in the standardization and further purification of insulin preparations. It is to be hoped that it will soon be feasible to decide some of the questions discussed in this paper with the aid of crystallized insulin, which has been recently isolated by Abel.[8]

NOTE: We wish to express our thanks to Eli Lilly and Co. and to the Connaught Laboratories, Toronto, for providing us with samples of insulin and with detailed information, regarding each.

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[7] *Cf.* Maiweg, H., *Biochem. Z.*, 1923, cxxxiv, 292. Frowein, B., *Biochem. Z.*, 1923, cxxxiv, 559.

[8] Abel, G. G., Pasteur lecture given before Chicago Institute of Medicine, January 22, 1926.

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Note on the adsorption of insulin by kaolin.

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Considerable progress has been made in the purification of enzymes since Willstätter and his coworkers have perfected the method of adsorption and elution.[1] It occurred to us that these methods might be successfully used in the further purification of insulin. Quantitative studies of the behavior of insulin in adsorption are difficult owing to the necessity of assaying the insulin physiologically. This difficulty, we believe, may now be overcome by the use of the iodometric method of estimation.[2]

This report deals with our studies on the adsorption of insulin by kaolin. The kaolin used in our experiments was a special brand, purified by electrodialysis.[3] The insulin preparation used (Lilly lot 769999, 20 units per mg.) contained 440 units per cc., and had an iodine value of 21.7 cc. 0.01 N iodine in neutral-buffered solution and of 34.5 cc. 0.01 N iodine in alakaline solution. The preservative was removed from the insulin preparation by isoelectric precipitation, because phenols interfere with the iodometric estimation. There were adsorbed 440 units of insulin on varying amounts of kaolin for 15 min. and centrifuged. The supernatant fluid is designated "adsorption rest-solution." The insulin was then removed ("eluted") from the kaolin with 0.01 N NH_3 and centrifuged. The insulin was precipitated from the supernatant fluid (designated "elution" by Willstätter) at pH 5 and centrifuged. The supernatant fluid is designated "elution rest-solution." The table shows our results on adsorption and elution, the percentages of insulin recovered from the solutions corresponding to the different steps in the procedure, and the iodine values from which the percentages of insulin were calculated. The amount and potency of the insulin in the adsorption

[1] Terminology according to Willstätter.

[2] Brand, E., and Sandberg, M., PROC. SOC. EXP. BIOL. AND MED., 1926, xxiii, 313.

[3] Wallstätter, R., and Waldschmidt-Leitz, E., *Z. physiol. Chem.*, 1923, cxxv, 132, p. 180.

INSULIN ADSORPTION BY KAOLIN.

Exper. No.	Adsorption of 440 un. at pH 3		Adsorption Rest-solution			Elution with 10 cc. of 0.01 N NH_3						Total I_2 value recovered.	
						Precipitate at pH 5			Elution Rest-solution				
	vol.	kaolin	cc. 0.01 N I_2 neutr.	cc. 0.01 N I_2 alk.	% insulin (I_2 absorbing material.)	cc. 0.01 N I_2 neutr.	cc. 0.01 N I_2 alk.	% insulin (I_2 absorbing material.)	cc. 0.01 N I_2 neutr.	cc. 0.01 N I_2 alk.	% insulin (I_2 absorbing material.)	neutr.	alk.
5	10 cc.	0.2 gm.	14.40	24.50	66*	4.80	7.90	22*	1.50	2.50	5	20.70	34.90
9	30 cc.	0.2 gm.	14.56	22.80	67*	6.70	11.35	31*	0.36	0.72	1.5	21.62	34.87
6a	10 cc.	0.4 gm.	7.20	10.10	33*	11.25	16.75	52*	3.18	6.30	15	21.63	34.40
7	10 cc.	0.6 gm.	1.40	1.80	6.5**	19.90	29.60	92*	0.36	1.35	1.5	21.66	32.75
8	10 cc.	0.8 gm.	1.40	1.80	6.5**	18.10	29.60	83*	1.84	1.68	8.5	21.34	33.08

*Tested physiologically and found active.
**Tested physiologically and found inactive.

rest-solutions and in the precipitates from the elutions has been roughly assayed physiologically as a check for the iodometric estimations.

Our experiments show that relatively large amounts of kaolin are required for the adsorption of insulin. Kaolin apparently is not a very specific adsorbent for insulin and it remains doubtful whether it can be used advantageously in the further purification of insulin preparations. Willstätter's special aluminum hydroxide preparations are sometimes extremely specific and selective adsorbents, depending on the type of preparation and the enzyme. Our preliminary experiments with aluminum hydroxide (preparations B and C according to Willstätter) seem to indicate that it is a more suitable adsorbent for insulin than kaolin.

Further experiments are necessary to establish more fully the behavior of insulin in adsorption and elution. It may be possible that such studies will lead to a practical method for the further purification of insulin.

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Glutathione in blood and its utilization in milk secretion.

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The work reported was originally an attempt to follow free cystine in blood by means of the Folin-Looney[1, 2] method. It was planned, if possible, to repeat with cystine the work done in this laboratory with tryptophane[3, 4] and thus to follow its utilization by the mammary gland in milk secretion, and to study further the changes that may occur in the composition of the blood mixture of amino acids as a result of various changes in diet.

Protein free blood extracts were made as in the amino N de-

1 Folin, O., and Looney, Joseph M., *J. Biol. Chem.*, 1922, li, 421.

2 Looney, Joseph M., *J. Biol. Chem.*, 1922, liv, 171.

3 Cary, C. A., and Meigs, Edward B., *J. Agr. Res.*, 1924, xxix, 603.

4 Cary, C. A., *Proc. Am. Soc. Biol. Chem.*, Dec., 1925.

terminations in this laboratory.[5, 6] These extracts gave values with the Folin-Looney method equivalent to 4 to 7 milligrams of cystine per 100 cc. of blood, and 85 per cent of this occurred in the corpuscles. This was much higher than expected. Furthermore, when cystine was added to the original blood it was never recovered quantitatively. Of 4 mg. dissolved in the acetic acid to be used in the coagulation of the blood, only 1.36 mg. were recovered. When 100 cc. of the original blood were shaken for half an hour with 100 mg. of cystine, only 5 mg. were recovered. With the Folin and Wu[6] tungstic acid method larger amounts of this color producing substance were found in the blood, but there was no recovery of added cystine. Passing air or hydrogen sulphide through the blood for three hours before coagulation had no effect upon the color given by it or upon the recovery of added cystine. Other methods of deproteinization were tried. The results indicated that the color-producing substance in the blood was not cystine; although, when heated with alkali and lead acetate, it gave a precipitate of lead sulfide, and, after treatment with mild reducing agents, it gave the nitroprusside test.

We suspected that the substance might be glutathione, but Hopkins[7] had been unable to find it in blood plasma, and Tunnicliffe[8] had reported it to be absent from whole blood. They had used the nitroprusside reaction which is none too sensitive and which reacts only with the reduced form. Glutathione is oxidized readily in neutral or slightly alkaline solution; and, although Tunnicliffe showed that it is present mainly, if not entirely, in the reduced form in muscle, liver and yeast, it might well occur largely in the oxidized form in the blood where apparently no precaution was taken to reduce it. We therefore decided to try to isolate it from the blood. Our first attempt failed, although some of the substance giving lead blackening and the nitroprusside test was present up to the very last step.

At this time Abel and Geiling[9] reported the presence of labile S in their preparations of insulin. Best, Smith and Scott[10] had

[5] Cary, C. A., *J. Biol. Chem.*, 1920, xliii, 477.

[6] Folin, O., and Wu, Hsien, *J. Biol. Chem.*, 1919, xxxviii, 81.

[7] Hopkins, F. G., *Biochem. J.*, 1921, xv, 286.

[8] Tunnicliffe, H. E., *Biochem. J.*, 1925, xix, 194.

[9] Abel, John J., and Geiling, E. M., *J. Pharmacol. and Exp. Therap.*, 1925, xxv, 423.

[10] Best, C. H., Smith, R. G., and Scott, O. A., *Am. J. Physiol.*, 1924, lxviii, 161.

shown the presence of this substance in blood. Although the preparations of insulin that we tried gave a faint Folin-Looney reaction, we were able to satisfy ourselves that it did not occur in our protein free blood extracts.

We then attempted to repeat our effort to isolate glutathione using a much larger quantity of blood than before. While this work was in progress Holden[11] published his paper describing the isolation of glutathione from blood. He actually crystallized out 5 mg. per 100 cc. of blood, which, considering the losses that would occur, would account for practically all the labile S that we found in the blood. Holden used the optical rotation of glutathione to guide him in its isolation, whereas we used the Folin-Looney reaction. Assuming the total reaction that we obtained to be due to glutathione, there would be approximately 10 mg. per 100 cc. of blood. This figure is not accurate. However, with some work the method may possibly be adapted to the quantitative estimation of glutathione in blood.

Early in our work we tried to determine whether the substance with which we were dealing, and which we may now call glutathione, is used in the secretion of milk. We compared its amount in samples of blood obtained simultaneously from the jugular and mammary (abdominal subcutaneous) veins of cows. These samples were practically identical when taken from dry cows; but, with lactating cows, the mammary samples showed approximately 25 per cent less than the jugular. That this difference is not due to a shift in the oxidation-reduction equilibrium between the two forms of glutathione is shown by our failure to influence our results by aerating or passing hydrogen sulfide through the blood. Apparently glutathione is used in the secretion of milk, and thus performs another function besides that attributed to it by Hopkins. Assuming that the substance that is thus utilized in milk secretion is entirely glutathione, one may approximate roughly the amount of it that the mammary gland takes out of the blood daily. It is sufficient to account for all of the glutamic acid in the milk proteins and several times the total S content of the milk. We have no definite data as to the disposition of this excess of S. In the formation of milk fat from phosphatide Meigs Blatherwick and Cary[12] found the excess P returned as inorganic

[11] Holden, Henry Francis, *Biochem. J.*, 1925, xix, 727.

[12] Meigs, E. B., Blatherwick, N. R., and Cary, C. A., *J. Biol. Chem.*, 1919, xxxvii, 1.

phosphate to the plasma of the mammary blood, and this S might be oxidized and similarly returned as sulfate to the blood.

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Immunity to pneumococcus afforded rats by feeding them the germ.

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In December it was reported[1] that rats became resistant to pneumococcus injections if fed on the tissues of other rats which had been killed by the same organism. It was shown that such animals tolerated 1000 or more times the dose of pneumococci that proved fatal for control rats. It was suggested that the living germs present in the tissue being fed might be the real cause of this increased resistance. Attention was also called to the fact that experiments had been done in which pneumococci were fed with the object of seeing whether a similar immunity could be built up in this manner. Our experiments at that time indicated that feeding the cocci from 50 cm. of culture per day to a rat produced a decided degree of immunity. It is our intention in the present report to give a representative experiment (our latest), in tabular form and to show that an immunity almost as good as, if not quite equal to that produced in the tissue feeding experiment, can be produced by feeding live pneumococci. Tests have also been made in which the dead germs have been fed, and are included in the table. The immunity built up by this latter method, is not so good as that obtained with living organisms, when fed in equal quantities.

Each of the rats listed in the accompanying table received the germs from approximately 50 cm. of a 24 hour culture of pneumococcus Type I, daily, for about 20 days. The organisms were grown in meat infusion media. The culture was centrifuged, the

[1] Ross, Victor, PROC. SOC. EXP. BIOL. AND MED., 1925, xxiii, 183-185.

germs suspended in 0.1 per cent gelatin solution, stirred, and cracker meal added and mixed. This was spread out on a shallow dish and given to the rats several hours after the last meal. In the experiments with dead germs, the suspended pneumococci in gelatin solution were heated to 80° C. for two hours, then cultures were made, and, if no growth occurred after 48 hours incubation, the material was fed after mixing with cracker meal. The feeding of live and dead pneumococci was carried on simultaneously. The two groups of rats were kept in separate cages. The weights of the rats increased at the usual rate and the animals were as active as controls.

In order to test the resistance of the animals, they were injected intraperitoneally with pneumococcus, Type I. The volume injected was in all cases 0.20 cm., although the actual number of organisms varied. A 24 hour broth culture was used. In the table are given the number of the rat, whether it is control or experimental animal, the kind of germs fed, *i. e.,* living or dead, the weight, the quantity of culture injected, and the result.

The control rats die of 0.00001 cm. and 0.000001 cm. Of the rats which were fed live germs, only one died of 0.0001 cm. Such rats generally survive 0.001 cm. and 0.01 cm. Of the rats fed on the germs killed by heat, none survived 0.001 cm. Some survived 0.0001 cm. although quite as frequently they died of this amount and even of 0.00001 cm. There is thus clear indication of the immunizing effect of the live germs and evidence for some effect of the dead germs. Experiments are in progress in which larger amounts of dead germs are being employed, with the object of seeing whether an immunity as good as that produced by the living organisms can be created. It is not unlikely that the method of killing the germs is important in determining their immunizing properties. Different methods of doing this are therefore being tried. Avirulent germs are also being fed. No suggestion as to the manner in which immunity by feeding is created is offered. Our earlier germ feeding experiments showing the effects of increasing quantities will be published in detail shortly.

The work of Killian[2] and of Eguchi[3] has just come to our notice. The former found, in a small group of mice, that heat

[2] Killian, H., *Z. f. Hyg., u. Infektionskrankh.,* 1924, cii, 279-286.

[3] Eguchi, Ch., *ibid.,* 1925, cv, 74-90.

Table Showing Increased Resistance to Pneumococcus of Rats which were fed Pneumococci

*Indicates a new day.

Rat No.	Control or Experim'tal	Germs fed. Live or dead	Wt. gm.	Quantity Injected cm.	Result D=dead
*—	C	—	103	2.10^{-3}	D. 1 day
227	E	L	109	2.10^{-3}	D. 1 day
239	E	D	103	2.10^{-3}	D. 1 day
*—	C	—	113	10^{-5}	D. 2 days
—	C	—	123	10^{-4}	D. 1 day
222	E	L	125	10^{-5}	Survived
214	E	L	125	10^{-4}	D. 2 day
250	E	D	125	10^{-5}	Survived
247	E	D	130	10^{-4}	Survived
*—	C	—	120	10^{-6}	D. 1 day
—	C	—	127	10^{-5}	D. 1 day
*—	C	—	113	10^{-5}	D. 1 day
—	C	—	123	10^{-4}	D. 1 day
*—	C	—	137	10^{-5}	D. 4 days
218	E	L	132	10^{-4}	Survived
217	E	L	141	2.10^{-3}	Survived
249	E	D	127	10^{-4}	D. 2 days
236	E	D	151	2.10^{-3}	D. 2 days
*—	C	—	142	10^{-5}	D. 2 days
215	E	L	145	10^{-3}	D. 2 days
225	E	L	170	10^{-2}	D. 2 days
252	E	D	155	10^{-4}	Survived
246	E	D	133	10^{-4}	D. 4 days
*—	C	—	145	10^{-5}	D. 2 days
—	C	—	143	10^{-5}	D. 2 days
229	E	L	158	10^{-4}	Survived
220	E	L	143	10^{-4}	Survived
232	E	L	153	10^{-3}	Survived
230	E	L	160	10^{-3}	Survived
238	E	D	127	10^{-4}	D. 2 days
242	E	D	146	10^{-4}	D. 2 days
237	E	D	166	10^{-3}	D. 2 days
234	E	D	165	10^{-3}	D. 1 day
*—	C	—	125	10^{-6}	D. 2 days
—	C	—	135	10^{-5}	D. 2 days
215	E	L	157	10^{-3}	D. 2 days
219	E	L	151	10^{-3}	Survived
224	E	L	174	10^{-3}	Survived
221	E	L	175	10^{-2}	Survived
240	E	D	140	10^{-5}	D. 3 days
253	E	D	191	10^{-4}	D. 2 days
235	E	D	175	10^{-4}	D. 2 days
245	E	D	160	10^{-4}	D. 2 days
*—	C	—	125	10^{-6}	D. 3 days
—	C	—	132	10^{-5}	D. 3 days
231	E	L	130	10^{-3}	Survived
228	E	L	145	10^{-3}	Survived
223	E	L	140	10^{-3}	Survived
220	E	L	172	10^{-2}	D. 2 days
216	E	L	180	10^{-2}	Survived
244	E	D	130	10^{-5}	D. 2 days
243	E	D	157	10^{-4}	Survived
248	E	D	154	10^{-4}	D. 2 days
241	E	D	142	10^{-4}	D. 2 days
251	E	D	173	10^{-3}	D. 2 days

killed pneumococci, when orally administered, were valueless as an immunizing agent and that the living germ was only slightly better. Eguchi finds that *young* mice can be immunized using dead pneumococci.

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The histology of local streptococcus immunity.

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The two most significant results of our studies over several years on localized streptococcus infections in the rabbit would seem to be, first: That, under properly controlled conditions, local infection is followed by a local form of immunity;[1, 2] and second, that local protection in the pleural cavity, whether in a form of increased resistance (broth) or of specific active or passive immunity, is associated with an increase in the number of clasmatocytes or tissue macrophages there present.[2, 3] A third study would associate clasmatocytes with the formation of antibodies.[4]

Repeated attempts to simulate conditions in the body by the action of clasmatocyte exudates outside the body have been confusing. The transfer of the entire pleural exudate of a clasmatocyte type, from a broth-protected to a normal animal, transfers no protection. Exudates of both polymorphonuclear and clasmatocyte type do not in their entirety destroy even a minimal number of streptococci in the test tube, although it may be shown that the supernatant fluids of both exudates are bactericidal, and furthermore, that the acid cell extracts of both types of cell will destroy streptococcus. (Unpublished observations.) But these extracorporeal phenomena do not account for the occurrences in the animal body, since they are much slower in effect, and, since the polymorphonuclear exudate yields more highly bactericidal

1 Gay and Rhodes, *J. Infect. Dis.*, 1922, xxxi, 101-115.
2 Gay and Morrison, *J. Infect. Dis.*, 1923, xxiii, 338-367.
3 Gay and Clark, *J. Infect. Dis.*, 1925, xxxvi, 233.
4 Gay and Clark, *J. Am. Med. Assn.*, 1924, lxxxiii, 1296.

substances (owing to the greater number of cells present) than does the clasmatocyte exudate. The former, however, as it occurs in the body, is associated with no protection of the animal.

When either stock or passage cultures of our streptococcus are injected into the protected pleural cavity of a rabbit, they disappear with great rapidity, and, under proper conditions the cavity is sterile within three hours. This disappearance is judged, not only by a diminished number in cultures but by actual decreased numbers of organisms in smears, when large numbers are used. Drainage from the pleural cavities either of dyestuffs, inert particles, or bacteria, is through the parietal pleura and diaphragm.[5,6]

We now find that the various degrees of protection, increased resistance and active immunity, are associated with remarkably pronounced changes in the pleura, both visceral as well as in the parietal pleura and diaphragm, but in the latter localities are more marked. The normal parietal pleura of the rabbit measures on the average 27 micra in thickness. Following injection of aleuronat (polymorphonuclear exudate), this thickness is doubled, owing to the infiltration of large numbers of polymorphonuclear cells. Here no protection is evident. Three days after the injection of aleuronat or of broth, the pleura is still further thickened, average 155 micra (x5); the polymorphonuclear cells are replaced by mononuclears, and protection is evident. These mononuclears by vital staining are shown to include a relatively large number of clasmatocytes as well as fibroblasts. Repeated injections of streptococcus, either in the broth prepared cavity or beginning with a stock culture, still further increases the pleura thickness, so that in one instance it reached over 1100 micra (x40). It presents all the appearances of granulation tissue.

So far as we have gone the degree of protection is paralleled by the increasing thickness of the pleura and increased number of clasmatocytes. Active phagocytosis of the streptococcus is confined in such tissue to the clasmatocytes lying under a relatively intact layer of serosal cells, which seems to explain best the mechanism of disposal of microorganisms in this form of localized immunity. We do not for a moment assume that protection even of this local type is entirely accounted for by a mere quantitative increase of the number of cells. There is probably a

[5] Gay and Rhodes. *J. Infect. Dis.*, 1921, xxix, 217.

[6] Karsner and Swanbeck, *J. Med. Res.*, July-Sept., 1920, xlii, 91.

specific factor, such as the local formation of antibodies or local mobilization of antibodies which cooperates in the process. This question and many others are being investigated in pursuit of the observations already stated, which we believe offer a most promising and concrete introduction to an histology of immunity.

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Active and passive protein sensitization in utero.

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It seemed probable to one of us that protein sensitivity in certain cases of early infancy might have some relationship to prenatal conditions.

Time does not permit our going into a detailed discussion of our hypothesis, nor can we enter into the clinical considerations, nor historical background for our work. We would merely like to present certain investigations on guinea pigs, which may have a bearing on this problem.

There has been a small but clearly defined amount of work by Rosenau and Anderson,[1] Anderson,[2] Gay and Southard,[3] Wells,[4] and others, on the passive transfer of antibodies from mother to offspring.

In the study of 29 guinea pig families, in which the mothers had been injected with normal horse serum long before conception, we induced acute anaphylactic death in the offspring born of these mothers, by an injection of normal horse serum within the first twenty-four hours to a few days after birth, thus corroborating the work of others.

* This work is being carried on under "The Crane Fund for the Study of Anaphylaxis."

1 Rosenau, M. J., and Anderson, J. F., *Hyg. Lab. Bull.*, 1906, xxix, 73.

2 Anderson, J. F., *J. Med. Res.*, 1906, x, 259.

3 Gay, F. P., and Southard, E. E., *J. Med. Res.*, 1907, xi, 143.

4 Wells, H. G., *J. Inf. Dis.*, 1911, ix, ii, 147.

We have also shown this passive sensitivity of the offspring to persist as a rule for 78 days, and in one instance, for 118 days. Thus for the present, we may assume that passive sensitization from mother to offspring generally lasts for about 2½ months, but may persist for even longer than 4 months.

Up to the present state of our investigation, we have shown this transfer to pass successively into the offspring of the second, third, and fourth litters, the mothers having received a single injection of horse serum before the first confinement.

In 15 families, in which the mothers were sensitized during pregnancy, the offspring, as in the former cases, showed sensitization at birth. We have not as yet demonstrated the influence that the antigen might have on the duration of sensitization in these offspring. This is under further consideration.

The foregoing experiments serve as a background for our present work. We believed, that if we could actively sensitize a mammal in the uterus of a mother, non-sensitive at the time of confinement, the experimental proof thus obtained, might be more in accord with the mechanism which may be present in certain of the remarkable instances of those infants born of non-sensitive mothers, who manifest profound anaphylactic symptoms when they ingest some protein food for the first time.

We have been unable to find any record in the literature of an attempt to clearly differentiate a passive transfer of antibodies from mother to offspring, from an active sensitization of a fetus *in utero*.

In order to accomplish our purpose, we attempted to so time the injections into the mother before confinement, as to obviate with certainty, the possibility for the establishment of antibodies before the birth of the offspring.

This was a difficult task, as one cannot easily time the date of parturition. It was therefore a question of guessing as nearly as possible, the end of a period of pregnancy, and thus make our injections within a few days before confinement. For example, we had 11 animals injected from 1 to 4 days, 18 that were injected between 6 and 10 days, and others which were injected a longer period before confinement.

It is obvious that when an animal was born within 1 to 4 days after the mother had been injected, no antibodies could have developed and been transferred to the offspring. However, in all instances, further evidence of a definitely non-sensitive state of

a family was obtained, by proving that either the mother or one of the offspring was not sensitized at the time of confinement. The remaining, or uninjected offspring, were then permitted to live for a month or more. This period of time, we believe, would suffice for the active development of antibodies—the direct result of contact of the fetus with the horse serum transferred from the mother's circulation through the placenta.

Not knowing the influence that an injection into the mother might have on the sucklings, we did not in every case, inject the mother after confinement, although in 10 families we have shown no evidence of transmission of antigen through the milk. This question is under further investigation.

The overwhelming number of these experiments resulted in an inability to produce this active sensitization *in utero*. These negative experiments total 26 families. In 8 families we obtained moderately suggestive results. In 3 families we obtained results that were fairly definite but not conclusive. In 1 family the results were very striking, but the period of injection prior to the confinement seemed too long to fit in with the criteria laid down above.

In view of the irrefutable fact, that antibodies pass through the placenta with regularity, the negative results that we obtained in the latter group of experiments, may be due to many factors, which at the present time cannot be answered. Among these considerations, there is the question of the length of time that it takes antigen to pass through the placenta, the question of whether this passage occurs in every instance, and the question of the ease with which the fetus is able to develop an active sensitization.

However, we believe that we have in two instances, definitely shown that a fetus can be actively sensitized *in utero,* and present the following two experiments as proof.

Mother 541, on 10/23/25, received a 10 cc. intravenous injection of horse serum. On 10/25/25, which was exactly two days after the injection of horse serum, this mother gave birth to two offspring. On 10/30/25, one of the offspring, five days after birth, or seven days after the mother had received her injection, was given an intravenous injection of 1 cc. horse serum. This animal remained perfectly normal. The second, or remaining offspring, on 11/20/25, was thirty-five days old, when it also received an intravenous injection of 1 cc. horse serum, and

promptly died in acute anaphylactic shock, presenting typically anaphylactic lungs.

In this instance, it seems clear, that the initial injection to the offspring, must have in reality been the second or toxic dose to the primary sensitizing dose it had received *in utero*. The negative reaction of the first offspring, in face of the overwhelming evidence of anaphylactic death, occurring in newly born guinea pigs of sensitized mothers, indicates very strongly that the sensitivity developed by the second offspring was that of an active production of antibodies.

Mother 310, on 5/19/25, received 2 cc. of horse serum intraperitoneally. On 5/22/25 (three days later), gave birth to one offspring. As long as fifteen days after the mother's first injection, *i. e.*, 6/5/25, we were fortunately able to show that this mother remained perfectly normal, after a 1 cc. intravenous injection of horse serum. The offspring was then permitted to live for twenty-eight days, and on 6/19/25, was given an intravenous injection of 1 cc. horse serum, and promptly died in acute anaphylactic shock. On this same day (6/19/25) the mother received her third injection of 1 cc. intravenously, and also promptly died in acute anaphylactic shock. This lends further evidence to the fact that on 6/5/25, the negative response of the mother to the second injection shows that she gave birth to her offspring before she was sensitized. (Three days as noted above is generally regarded as too short a time for the development of antibodies.)

These facts obtained in guinea pigs, we hope may suggest some possible explanation for certain instances of anaphylactic reactions in early childhood, when these individuals ingest some foreign protein for the first time. We are engaged in further studies on this entire problem.

2960

Further studies on a specific pneumococcus toxin.

J. G. OLSON. (Introduced by G. H. A. CLOWES).

[*From the Lilly Research Laboratories, Indianapolis, Ind.*]

In a previous preliminary report[1] on a specific pneumococcus toxin and corresponding antitoxin, the cutaneous reactions incited by the intracutaneous injection of such toxin, and the lung pathology produced by its intraperitoneal or intravenous injection, have been pointed out. Larson[2] has also reported his results in applying intracutaneous tests to a small series of human beings and has confirmed our observations on the effect of pneumococcus toxin on mice. This paper will report further preliminary observations we have made.

Dr. E. Vernon Hahn is collaborating with us in a study of the pathology produced in animals by pneumococcus toxin. Detailed reports of histologic studies of its effects upon various organs, particularly lungs, liver, kidneys, and spleen, will be made later.

Since it has been found possible to estimate the strength of various lots of both toxin and antitoxin on the basis of the ability of the toxin to produce lung pathology, and of the antitoxin to prevent it, when the two are injected together, a few brief descriptions will be given:

Pneumococcus toxin given to mice in lethal or slightly sublethal doses, has been found to produce a more or less characteristic gross and histologic picture. Grossly the lungs show edema, engorgement, and frequently apparent massive consolidation, with hemorrhage.

On section intense congestion and interstitial hemorrhage with decreased air content of the alveoli have been noted. There is inter-alveolar extravasation but no extensive filling up of alveoli with exudate or blood, that is, the processes are patchy. An inflammatory or fibrinous exudate may be found in the bronchioles, with or without marked degeneration and vacuolization of the bronchiolar epithelium. Polymorphonuclear leucocytes may be found grouped within and often adherent to the endothelium of

1 PROC. SOC. EXP. BIOL. AND MED., 1926, xviii, 294.

2 *Ibid.*

the blood vessels. Varying degrees of interstitial pneumonitis with pigment-laden macrophages scattered throughout the parenchyma have been noted. There may be a more or less notable catarrhal reaction in the bronchioles and alveoli with degeneration of the alveolar epithelium. Varying degrees of edema just beneath the pleura have been noted. This description relates to animals that have received interaperitoneal injections of sterile pneumococcus toxin. Numerous cultures made at autopsy have proven sterile.

In contrast with this, the lungs of animals that have received broth alone and subsequently been killed by chloroform vapor, usually show hyperemia only, with little or no exudate in the alveolar walls, which may sometimes show slight edema.

Approximately the same result as from broth alone is obtained by the injection of heat-destroyed toxin.

Proof that pneumococcus antitoxin prevents lung pathology is subject to statistical interpretation. There may be little or no gross evidence of injury, yet on section the lungs may show some catarrhal reaction, and patchy pneumonitis. They commonly lack, however, the definite polymorphonuclear reaction, the congestion and degeneration met with when toxin alone has been given.

Our experiments indicate that neither the skin reaction in rabbits nor the characteristic lung pathology we have mentioned can be induced by filtrates, in all respects similar to pneumococcus filtrates, excepting that they are prepared from *M. aureus, S. viridans, S. hem. scarlatinae, B. mucosus capsulatus, B. coli or B. typhosus.*

That we are working with an exotoxin and not an autolytic product or endotoxin is indicated by the fact that eight hour culture filtrates are found to possess marked specific toxicity, as measured by rabbit skin test and mouse lung pathology. The toxicity appears to rise sharply for about twenty-four hours, after which it has not ordinarily been found to increase, but rather to remain stationary or to decrease considerably. Five days is the limit of incubation time we have observed. Doubtless the substrate is an important determining factor. We regularly work with twenty-four hour filtrates. Toxin of 300,000 rabbit skin test doses per cc. has been prepared by twenty-four hours' incubation.

Pneumococcus toxin is relatively thermostabile, though perhaps less so than scarlet fever streptococcus toxin which it resem-

bles in this respect. We have completely destroyed the toxicity of filtrates by an hour's boiling. Simple coagulation of toxin-filtrate constituents has not been found to destroy the toxicity of the filtrate.

Heating pneumococcus toxin for short periods of time at relatively low temperatures seemingly heightens its activity, and it can apparently be destroyed by prolonged heating at temperatures under 100° C. Light appears to be detrimental. Unpreserved lots have been found to lose their toxicity with relative rapidity, whereas the addition of preservative, *e. g.,* 0.3 per cent cresol, has apparently prevented loss of potency over a period of several months, whether at ice-box, room, or incubator temperatures. Since 0.3 per cent cresol itself induces reactions in delicate viscera, we are obliged to use fresh filtrates or filtrates containing not more than 0.05 per cent cresol, to avoid confusing results, in some of our work. Dilution seems to favor loss of potency.

Samples of pneumococcus toxin have been tested and found to contain very little hemotoxin. Moreover, when the hemotoxin has been absorbed out, in the cold, and the absorbed filtrates compared with unabsorbed controls by rabbit skin tests, no quantitative decrease in toxicity has been observed. An hour's combined shaking and aeration is likewise found to produce no measurable decrease in toxicity.

Preliminary observations by Mr. E. E. Swanson on a series of dogs, prepared as for ordinary pharmacologic respiration and blood pressure tracings, indicate that toxic pneumococcus filtrates produce a reduction of amplitude of the respiratory wave on a tracing. This is generally associated with cardiac acceleration and irregular respiration. The original culture medium, heat-destroying toxin, or toxin neutralized by corresponding antitoxin have not been found to produce such changes. Moreover, when a dog has been allowed to develop such toxemic manifestations, we seem to be able to relieve completely the respiratory distress, and to a less extent, that of the heart, by the administration of antitoxin.

Preliminary attempts at active immunization of mice by serial injections of pneumococcus toxin have resulted in protection against 1,000 to 10,000 fatal doses of virulent pneumococci as measured on normal controls injected simultaneously. Our experiments indicate that the immunity operates irrespective of

type. Partially immunized animals killed by an overdose of organisms have been found to exhibit intense hyperemia of the peritoneum and abdominal wall, with serosanguinous fluid in the abdominal cavity.

Normal sera from horses, sheep, rabbits and chickens have been titrated against pneumococcus toxin and have ordinarily been found to exhibit very little power, either to prevent cutaneous reactions in rabbits or lung changes in mice. The same is true of regular antipneumococcus sera or antibody solution, and such immune sera as antistreptococcus serum, scarlet fever antitoxin and diphtheria antitoxin.

Further work is in progress. A detailed account of some of our experiments will be published soon.

2961

On a specific pneumococcus antitoxin.

G. H. A. CLOWES, W. A. JAMIESON and J. G. OLSON.

[*From the Lilly Research Laboratories, Indianapolis, Indiana.*]

Specific pneumococcus antitoxins have been produced in this laboratory by various procedures, including those of Dochez, Larson, and the procedure developed in this laboratory by one of the authors (Olson), using a specific pneumococcus toxin.

The Larson procedure, which consists of injecting whole culture, appropriately attenuated, by means of a highly purified castor oil soap, has been extensively employed by us, using principally rabbits and sheep. Recently somewhat better yields of antitoxin have been obtained by injecting subcutaneously into rabbits, sheep or horses, progressively increasing doses of the sterile pneumococcus toxin.

The same pathological lesions were observed in the larger animals after the injection of successive large doses of toxin, as had previously been noted in smaller experimental animals and reported in a previous paper.

The highest concentration of antitoxin has been secured by starting with a very small amount of toxin and gradually increasing the size of the dose.

The procedure now adopted in testing each lot of antitoxin before using it experimentally on cases of human pneumonia, are as follows:

(1) *The rabbit Skin Test.* A series of dilutions of the antitoxin are mixed in equal proportions with a given concentration of the toxin, such that 0.1 cc. of the mixture contains twenty-five skin test doses of the toxin. The solutions in question are then incubated for a period of one to two hours and a considerable series of skin tests are carried out on a series of rabbits, using 0.1 cc. doses of the solutions in question. In this manner the amount of serum required to neutralize twenty-five skin test doses of toxin is determined. The law of multiple proportions appears to hold approximately for the neutralization of double or quadruple the number of skin test doses of a given toxin by a given antitoxin. We have adopted as a provisional temporary unit the amount of antitoxin required to neutralize one million skin test doses.

(2) *The Mouse Lung Test.* A similar series of dilutions of antitoxin are mixed in equal volumes with a fixed amount of toxin, so that 0.5 cc. of the mixture will contain approximately five thousand skin test doses of toxin. The mixtures in question are incubated for a period of one and one-half to two hours, and 0.5 cc. doses injected intraperitoneally into mice, a large number of duplicate tests being put on at each concentration. The test animals in question, with a series of controls which have received the toxin antitoxin, are killed at the end of 24 hours by means of chloroform vapor, if they have not already died as a result of the the toxin without antitoxin, are killed at the end of 24 hours by means of chloroform vapor, if they have not already died as a result of the toxin injection. A gross and if necessary a histologic comparison of the lungs are made, and from a statistical consideration of the data the approximate point at which the toxin is neutralized by the antitoxin is determined.

In comparing sera of varying antitoxic concentration, it is found that the variation between the skin test results is far less pronounced than that between the mouse lung results, and from a comparison of the data in question with such clinical results as are available to date, we are inclined to believe that the lung test will ultimately afford a more accurate index of the probable therapeutic value of the preparation than does the skin test.

(3) *Pharmacological Respiratory Tests.* A series of dilu-

tions of antitoxin are mixed in equal volumes with a fixed amount of toxin and after incubation for a period of 24 hours the solutions in question are injected intravenously into dogs. The determination of the point at which the antitoxin fails to inhibit the characteristic respiratory depression exerted by the toxin may afford a rough index of antitoxin value.

(4) Dr. H. M. Powell has obtained a flacculation reaction between the toxin and antitoxin which may afford an additional means of standardization.

(5) The antitoxic sera are tested as regards their effect on the temperatures of a series of rabbits with a view to affording some possible information regarding the presence or absence of chill-producing substances.

The pneumococcus toxins and antitoxins do not appear to be type specific. After carrying out a series of preliminary experiments on rabbits, a group of 25 sheep were immunized as follows: Five were given toxin from a Type 1 organism, five from a Type 2, five from a Type 3, five from a so-called Type 4, and five from a mixture of toxin derived from all four types.

After a suitable period of immunization the animals were bled and the individual groups were tested separately against all four toxins. While the antigenic value of the toxins produced from the cultures employed varied somewhat, and there was consequently a variation in the titre of antitoxin produced, it was found that with any one antitoxin its power to neutralize a given number of skin test doses of any one of the toxins employed was approximately equal.

On account of the difficulty invariably associated with the administration of large amounts of unconcentrated serum to cases of human pneumonia, an attempt has been made to purify and concentrate the pneumococcus antitoxin in the Chemical Division of the Lilly Research Laboratories. George B. Walden and Jasper P. Scott, working with one of the authors (Clowes), have succeeded in purifying the pneumococcus antitoxin to the extent of removing up to 99.9 per cent of the associated serum proteins, without appreciable loss of antitoxin when tested by the methods outlined above.

The antitoxin may consequently be concentrated from one to two thousandfold if so desired, but in practice it appears preferable to employ a twenty to one hundredfold concentration, which in its purest form gives a water-white, limpid, non-viscous solu-

tion. This purified antitoxin may be boiled for half an hour without suffering any appreciable loss of potency.

When injected intravenously in cases of human pneumonia the highly purified solutions do not appear to cause serum sickness. The chemical constitution of this highly purified pneumococcus antitoxin is being investigated at the present time in the Lilly Research Laboratories.

Experiments on the use of both unconcentrated and concentrated pneumococcus antitoxin in cases of human pneumonia have been in progress for a considerable period of time. The results obtained to date appear to justify further clinical experiments on a larger scale.

This conclusion is also supported by the interesting observation that more recently prepared antitoxic sera derived from animals which are now attaining a higher measure of immunity, exhibit strikingly increased protective effects when tested by the mouse lung method.

2962

Experiments on the development of the ear of amblystoma punctatum.

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A series of experiments was performed during the years 1922-1925 on embryos of *Amblystoma punctatum*. Two main lines of investigation were carried out, namely, experiments to determine the limits of the regenerative capacity of the tissue surrounding the normal ear region, and experiments regarding the nature of the developing ear itself.

The first group involved extirpation of ectoderm in the ear region of embryos at different stages of development. The size of the pieces removed was 0.2 mm., 0.3 mm., 0.4 mm., 0.5 mm., and 0.6 mm., respectively. It was found that complete regeneration generally followed when the operation was performed on stages earlier than that in which invagination of the ectoderm had occurred. Regeneration was completely checked only by the re-

moval of a piece of ectoderm 0.6 mm. in diameter, after a distinct auditory plate had formed.

A definite loss in the regenerative capacity of the surrounding ectoderm was evident, following invagination of the ear. The regenerated labyrinths were greatly reduced and, in several cases, no regeneration took place. Transplantation of undifferentiated ectoderm to the extirpated area caused a distinct lessening in regeneration in the earlier stages, and prevented it altogether in the later stages.

The second group of experiments dealt with the potencies of the ear at the time of invagination and at the time of closure of the vesicle. Experiments were performed involving removal of the anterior, posterior, dorsal and ventral halves of the ear respectively, at these stages, and it was found that while the remaining half, in 25 per cent of the cases formed a complete labyrinth, there was a tendency toward characteristic defects following each type of operation. These defects were more constant at the later stages of operation.

A series of experiments involving reciprocal transplants between white and pigmented embryos brought out clearly the fate of the different quadrants of the auditory plate. The endolymphatic duct and *macula sacculi* alone develop from the dorsal half; the canals, utriculus and the remainder of the sacculus, from the ventral half of the plate. This brings the point of closure of the vesicle very definitely at the base of the endolymphatic duct just where, later, the depression occurs between the duct and the dorsal wall of the utriculus. In the antero-ventral quadrant arises the sensory portion from which develop the *macula utriculi*, and the anterior and lateral cristae. The posterior crista undoubtedly occupies a similar position in the postero-ventral quadrant. These areas are at first apparently continuous with that portion in the dorsal half, which later forms the *macula sacculi*.

From these results it was concluded that at the time of invagination, the ear is in a transitional stage between a condition of equipotentiality and one of localization of definite organ-forming areas.

2963

Observation on hand sterilization.

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During the years 1917-1922, inclusive, fourth year medical students were instructed to prepare their hands for surgical operations by scrubbing for five minutes in running hot tap water and surgical soap, and by immersion of hands in one of the following germicidal solutions: alcohol, 70 per cent; alcohol, 95 per cent; bichloride and alcohol (Harrington); or by following the soap and water scrubbing by the use of lime and soda. The technic was in all practical respect identical with that in common use in the hospital operating-rooms. Cultures were taken after the scrubbing in soap and water, and after the use of one of the antiseptic solutions. The results follow:

Results of cultures taken from hands of medical students after soap and water scrubbing and use of antiseptic solutions.

	After soap-water	Alc. 70	Alc. 95	Bichl-Alc.	Lime-soda	Antisep. Sol.
Staphylococcus	90	15	3	13	6	39
Bac. coli	11	0	0	0	0	1
Bac. subtilic	2	1	0	2	0	1
Sterile	10	3	10	6	3	26
Percent sterile cultures	9	16	77	29	33	39

In interpreting the above data, allowance should be made for the placing of wet hands in the respective solutions. The reduction of the strengths of the solutions from this source applied particularly to the alcohols. "Antisep. Sol." indicates some one of the foregoing antiseptic solutions, the particular one not being clearly apparent from the records.

2964

Photopharmacology. IV. Effect of radiation on digitalis.

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The effects of various radiations on the potency and keeping qualities of digitalis were studied. The official tincture of digitalis was used in our experiments. The activity of the preparations was tested in two ways: on the one hand by the cat method, and, on the other hand by the new phytopharmacological method developed by the authors and described elsewhere.[1] The effect of ultra violet rays was studied with a Krohmayer water-cooled mercury vapor quartz lamp and with the Hanovia Alpine Sun Lamp. Tincture of digitalis was radiated with these machines in quartz containers and in pyrex and ordinary glass test tubes for various length of time. The effect of ultra violet radiation was to produce a rapid deterioration of the tincture. Radiation for one hour with the Krohmayer lamp or with the Alpine Sun Lamp of digitalis in a quartz tube produced as much as 30 per cent deterioration in some cases. An analysis of the various rays short and long, in respect to their activity on digitalis, showed that the shorter wave lengths, that is, those less than 2800 angstrom units were more active than the longer ultra violet rays. Digitalis in pyrex vessels was affected deleteriously to a greater extent than digitalis contained in ordinary glass containers.

In addition to the ultra violet rays obtained from the mercury lamp the effect of still shorter radiations on digitalis was studied. Samples of tinctures were exposed to x-rays, to radium directly and to radium emanations. It was found that x-rays and the radium radiations produced destructive changes in the tincture as far as pharmacological potency was concerned. The effect of heat radiations was also studied and it was found that exposure to temperature of 39° for 24 hours or longer, produced considerable deterioration in the tinctures which were tested by the methods described above. The effect of other infra red radiations is under investigation.

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[1] Macht, D. I., and Krantz, J. C., *J. Am. Pharm. A.*, 1924, xiii, 1115.

2965

On the ionic basis of electrical stimulation.

A. R. MOORE.

[*From the Physiological Laboratory of Rutgers University, New Brunswick, N. J.*]

In their attempts to develop a physical theory of electrical stimulation, Nernst[1] and A. V. Hill[2] have supposed the current to act by carrying the ions within the tissue against a membrane barrier. The resulting heaping up of the ions at the membrane constitutes the first step in excitation. If we assume, as the work of Loeb[3] shows we have a right to do, that the positive ions are the effective ones, then according to the views of Nernst and of Hill stimulation must occur at the cathode when the current is made. This is a statement of Pflüger's law and is valid for frog tissue, with which Pflüger worked.

A consideration of cases in which Pflüger's law is reversed suggests that a somewhat different and more general picture of the ion mechanism of electrical stimulation is necessary. In the ctenophores Beroe and Mnemiopsis, when the galvanic current is passed through a trough of sea water containing them, the result is a luminescent glow on the anodal side of the animal.[4] This glow occurs on the make and lasts for some seconds during the flow of the current. If an incision is made in the animal transversely with reference to the direction of the flow of the current, then anodal stimulation occurs also at the cut surface. These results mean that galvanic stimulation takes place only at the protoplasm-sea-water surface, and that stimulation is referable to the blocking of positive ions of the sea water at that surface. These ions, therefore, impinge on the protoplasmic membrane from outside. It is a remarkable fact that in ctenophores which consist so largely of included sea water there is no apparent stimulation as a result of internal ionic movements. This conclusion is necessary since stimulation appears only along the surface of the ani-

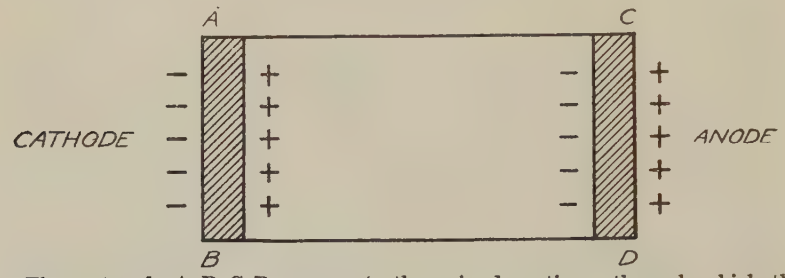
1 Nernst, W., *Arch. f. d. ges. Physiol.*, 1908, cxxii, 275.

2 Hill A. V., *J. Physiol.*, 1910, xl, 190.

3 Loeb, J., *Dynamics of living matter*, New York, 1906, p. 78.

4 Moore, A. R., *Am. J. Physiol.*, 1925, lxxii, 230.

mal next the anode. The situation may be represented by the figure. If stimulation is caused by ions inside the tissue impinging on the membrane, then excitation occurs at A B and is cathodal, *i. e.,* according to Pflüger's law. On the other hand, if ions outside the tissue produce excitation, then the effect is at C D and is anodal, *i. e.,* according to the reversed law of Pflüger. The latter is evidently the situation in ctenophores.



The rectangle A B C D represents the animal or tissue through which the current is passed as indicated by the terms "anode" and "cathode". In consequence, presumably ions would collect as shown on either side of the membranes AB and CD.

Electrical stimulation of the earthworm constitutes another type. Here stimulation occurs at the anode alone when a small current is used, but with stronger current cathodal stimulation takes place simultaneously with that at the anode. Thus at 6 volts a current of .025 ma. produces stimulation only at the anode, but .03 ma. causes excitation of the cathodal region also. Stimulation in the tissues of the earthworm, therefore, takes place as a result of the movement of ions both within and without the tissue, *i. e.,* at both A B and C D in the figure.

There are then three cases as regards the ionic basis of electrical stimulation. (1) The classic case of Pflüger, in which the ions of the tissue stimulated impinge on the membrane from the inside, *i. e.,* at A B, in the region of the cathode. (2) The case of the reversed law of Pflüger, in which the ions in the outside medium alone are effective and produce their action at C D, on the anodal side. (3) Where both types of stimulation occur, both the ions inside and those outside the tissue are effective in causing stimulation, as at A B and C D.

2966

On the heterogenetic haptene.

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In a previous communication[1] it was stated that active fractions of the specific part of the heterogenetic antigen, haptene, were obtained by fractional precipitation with alcohol from a solution in chloroform. The material obtained in this manner was extracted with hot alcohol. The residue was dissolved in water. On the addition of an equal volume of alcohol a precipitate formed. This precipitate was further dissolved in pyridine at 60° to 70° C. A smaller insoluble part was removed (although it also was active) and from the remaining solution, on addition of acetone, the material settled out. This product was soluble in water, unlike the known cerebrosides. It was practically insoluble in ether, acetone and cold[2] alcohol. On shaking with absolute alcohol at room temperature a little more than one part in 1000 went into solution. In hot alcohol it was soluble to the extent of about ½ per cent. While at first it did not seem possible to make a filterable chloroform solution, a colloidal solution in chloroform (strong Tyndall phenomenon) could now be obtained from which the substance did not separate on cooling or on concentration to a small volume. From a chloroform solution of the substance very little passed into water on shaking and conversely, chloroform extracted very little of the substance from an aqueous solution.

A 5 per cent solution of the product in water gave a rotation of approximately $[\alpha]_D = +20°$, in pyridine $[\alpha] = +10°$. With orcinol, hydrochloric acid and copper sulfate or ferrous sulfate the preparation gives a purple color.

On heating with ½ normal hydrochloric acid from 5 to 15 minutes a precipitate is formed. At the same time the serological activity is almost entirely destroyed. The soluble part, serologically inactive, reduces Fehling's solution, gives a strong purple

[1] Landsteiner, K., and Levene, P. A., *J. Immunol.*, 1925, x, 731.

[2] In the former communication the word cold was omitted.

color with orcinol, hydrochloric acid and copper sulfate, but it gave very little or no crystallized osazone with phenylhydrazine. Upon further heating of the acid solution, black products are formed, the solution yielding a crystallized osazone.

If the insoluble part resulting from the treatment with hydrochloric acid is heated further with new portions of acid, more reducing sugar is formed. The final solutions gave with orcinol and copper sulfate a green color instead of the purple. These solutions were dextrotatory and gave a crystalline phenylosazone. The insoluble material resulting from complete cleavage contained higher fatty acids and a base resembling sphingosine.

From beef kidneys, not containing heterogenetic antigen, only an insignificant quantity was obtained of a material similar in its solubility to the above. In a second experiment, such a product was not obtained.

Aside from the fraction described others were found which were similarly active, were water soluble, but gave a weak orcinol-copper test.

2967

Further studies on the production of staphylococcus aureus toxin.

JULIA T. PARKER, J. GARDNER HOPKINS and ANNE GUNTHER.

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In a previous paper[1] we showed that certain strains of *Staphylococcus aureus* produce an exotoxin which causes necrosis when injected intradermally into rabbits. With the methods used at that time only 8 out of 118 strains from pathogenic sources produced a toxin of sufficient strength to be demonstrable by this method. Recently, however, by two improvements in method, it has been possible to obtain the toxin from 13 out of 14 strains. These changes were: first, the growth of the staphylococcus culture in an atmosphere containing 10 per cent carbon dioxide and,

[1] Parker, Julia T., *J. Exp. Med.*, 1924, xl, 761.

second, the use of "Proteose peptone"* instead of Witte peptone in the broth media.

For cultivation in the presence of CO_2, the method of Cohen and Fleming[2] was used. The stronger toxin production in this atmosphere may be due to the fact that the pH of the broth cultures does not rise above 7.6 even after 8 days growth. When grown under the usual atmospheric conditions, even after 3 or 4 days growth, the staphylococcus cultures have a pH of 8 or over.

There appeared to be some variation in different strains, but a strong toxin was produced most regularly when "proteose peptone" was used.

In the production of other bacterial toxins it was found the amount produced by the staphylococcus varied greatly when different commercial brands of peptone were used in the medium.

The table is given to show the amount of toxin which seven different strains of *staphylococcus aureus* produced on media prepared in exactly the same way, except for the presence of 6 different peptones. All of the cultures were grown in the same jar with 10 per cent carbon dioxide at 37° for 3 days.

PEPTONES.

Strain No.	W	P	Proteose	C	F	B
169	0	0	+++	++	0	0
170	0	±	++	+	±	0
174	±		++++	++	+	++
175	±		+	++		+
176	0		++	+++		0
177	++		+++	+		+++
180	+++		+++	+++		

The toxins were tested in the usual way; namely, 0.1 cc. of a sterile Berkefeld filtrate of each was inoculated intradermally into rabbits. 0 indicates no reaction; ±, red reaction 2 by 2 cm. lasting at least three days; +, yellow center 2 by 2 cm. surrounded by erythema; ++, yellow center 3 by 3 cm. surrounded by erythema; +++, yellow center 3 by 3 to 4 by 4 cm.; ++++, yellow center 4 by 4 cm. to 6 by 6 cm.

For the convenience of those who may wish to make staphylo-

* "Proteose Peptone" was obtained from the Digestive Ferments Co., Detroit, Michigan.

[2] Cohen and Fleming, *J. Infect. Dis.*, 1918, xxiii, 337.

coccus toxin, the method we have used for preparation of the medium is given in detail.

Two pounds of chopped veal is placed in one liter of distilled water and left in the ice box over night. The infusion is then strained through cheese cloth, boiled, filtered and the reaction adjusted to pH 7.4. After autoclaving, the infusion is inoculated with *B. coli* and incubated for 18 hours. The broth is then sterilized in the Arnold, filtered, the volume made up to one liter, and 80 grams of "proteose peptone" (Digestive Ferments Co.) and 10 grams of NaCl are added. The whole is boiled in a double boiler for 40 minutes, filtered, the volume brought to one liter and the pH adjusted to 7.4, one liter of phosphate buffer [N/15 ($Na_2H\ PO_4$ and $KH_2\ PO_4$)] pH 7.4 is added, the media is distributed in Ehlermeyer flasks and autoclaved.

On this media in the presence of 8 to 10 per cent CO_2, the strongest toxin is obtained after 6 to 8 days' growth of the organism.

2968

The occurrence of scarlet fever without a rash during epidemic.

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During the winter of 1924-25 an epidemic of scarlet fever and acute streptococcus pharyngitis occurred among the nurses at Presbyterian Hospital, New York City. Early in the epidemic the entire nursing staff was tested for susceptibility to scarlet fever by means of intracutaneous toxin injections. Following this series of skin tests cultures were obtained from all throats showing an angina. Practically all of the cases of pharyngitis and tonsilitis showed hemolytic streptococci. The strains of hemolytic streptococci recovered from these cases were tested for agglutination with scarlatinal immune sera and with sera pre-

pared with two of the throat strains proven not to be scarlatinal strains. Filtrates were prepared from cultures of all these strains and tested for the presence of toxin by means of cutaneous reactions in Dick positive individuals. The presence of toxin was determined by heat lability (100 degrees C. for 2 hours), and by neutralization with scarlatinal antitoxic sera.

Twenty-three strains of hemolytic streptococci were studied. Six strains, which were obtained from cases of clinical scarlet fever, produced a heat labile toxin which was neutralized by anti-scarlatinal sera. Five of the strains were agglutinated by two scarlatinal immune sera, and the sixth, although it did not agglutinate, absorbed the agglutinin from these sera for other scarlatinal strains. This strain was apparently physically incapable of agglutination, yet was similar antigenically to the other scarlatinal strains. These strains (six) include all the strains from clinical scarlet fever.

The remaining seventeen strains were obtained from cases of pharyngitis and tonsilitis in which no rash occurred. Five of these strains produced toxin which was heat labile and was neutralized by scarlatinal antitoxin. As far as biologic characteristics are concerned these strains are scarlatinal strains, since they produced toxin and likewise were found to agglutinate and absorb agglutinin with the two scarlatinal immune sera. Four of the individuals from whom the strains were obtained were Dick negative previous to their throat infection. The fifth had not been tested.

The majority of the remaining twelve strains (9) agglutinated in immune sera prepared with non-scarlatinal strains. There were apparently two groups of strains represented by these sera as well as some additional groups, but the reactions were not strongly positive with these sera so we can only conclude that the strains were not antigenically similar. Apparently the strains from these cases were not bound as closely together as were the strains from the cases of scarlatina. One strain produced a heat labile toxin which could not be neutralized.

We conclude that throat infections with scarlatinal streptococci may occur without a rash. In this epidemic they occurred in individuals who showed negative Dick reactions and were presumably immune. In general, toxin production and agglutination of scarlatinal strains were parallel. Considering the agglutination reactions with all of the sera and all of the strains we conclude

also, that no antigenic likenesses exist between strains of streptococci from acute throat infections similar to that observed between scarlatinal strains.

2969

Unusual instances of infection with streptococcus scarlatinae.

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In the preceding study we noted that infections of the throat with scarlatinal streptococci may occur during an epidemic of scarlet fever, and that these infections are not accompanied by a rash. There is also clinical and epidemiological evidence that these cases may be responsible for cases of clinical scarlet fever among contacts. Williams has recently found this streptococcus in osteomyelitis, endocarditis and in chronically inflamed tonsils, so that we may be assured that *Streptococcus scarlatinæ* is a rather widely distributed organism and is not confined in its distribution to the cases diagnosed as clinical scarlet fever.

We know little concerning the clinical manifestations of *Streptococcus scarlatinæ* in conditions other than the usual angina with the scarlatina-form rash because the methods of identification are so recent that few observations have accumulated. Some of the infections are associated with a rash as in wound scarlet fever, yet we know that the infection may take place without cutaneous manifestations. The original strain for the toxin made by Dick and Dick was obtained from the infected finger of a nurse caring for a scarlet fever patient. In this instance no rash was reported.

The study of such atypical infections is important from the epidermiologic standpoint. Unfortunately, there is but little opportunity to study such cases in infectious hospitals because they are seldom recognized unless they occur among known scarlatinal contacts, or unless they are uncovered in an attempt to account

for a case of scarlet fever without known exposure. During the past two years we have been able to collect data on six cases of infection with *Streptococcus scarlatinæ* which did not present the usual rash, yet occurred among contacts of scarlatina or were apparently the source of scarlatinal infection in others. The identification of the strains was made by their toxin which was neutralized with antiscarlatinal serum. These data are presented briefly in the following paragraphs:

CASE *I.* The head of a family returned home after an extended vacation. He developed an acute coryza soon after his arrival. A few days later his wife developed a mild pharyngitis and antrum infection with considerable nasal discharge. The daughter developed clinical scarlet fever a few days after the mother was taken ill. She responded to antiscarlatinal serum. Cultures from the nasal passages of the mother showed streptococci producing scarlatinal toxin. The Dick test was faintly positive. Serum was given as a prophylactic and the pharyngitis subsided immediately. She had had no rash. She was probably the source of the scarlatinal infection in the child.

CASE *II.* The head of a family was severely prostrated with an acute angina. Throat cultures showed two types of hemolytic streptococci morphologically different on blood agar plates. One of these streptococci was later found to be a *Streptococcus scarlatinae.* The father had showed no rash. His Dick reaction was negative on the fourth day. Previous reactions were not done. Three days after he became ill a daughter, aged three, developed clinical scarlet fever. She had not been exposed to scarlet fever, since she had been in contact with no one except the mother and a nurse for the previous two weeks. The father apparently had a mixed streptococcus throat infection. What part the scarlatinal streptococcus played in the infection we are not certain. We must conclude, however, that he was responsible for his daughter's infection.

CASE *III.* Two children developed scarlet fever within a period of 48 hours. They were given antiscarlatinal serum on the fourth day with marked therapeutic benefit. The mother who was Dick negative and had refused prophylactic serum developed an acute streptococcus pharyngitis seven days after the children were first taken ill. The culture of this streptococcus was

lost in the laboratory before the toxin production could be determined. The bacteriologic evidence is not complete in this instance since we did not determine that the strain from the mother was a scarlatinal strain; however, she had had no outside contact for seven days previous to her illness and we conclude she had scarlatina without a rash.

CASE *IV*. Two children were in bed two weeks with an acute bronchitis. After the second week one of the children developed an acute otitis media with discharge. Two days later the sister vomited and twenty-four hours later showed a typical scarlatinal throat, tongue and rash. Scarlatinal streptococci were recovered from the ear of the first patient and from the throat of the second patient. Since there had been no known scarlatinal contacts, it was concluded that the first child had a scarlatinal otitis media without a rash, and that she was responsible for the infection of her sister. No skin reactions were done to determine if she developed an immunity subsequently.

CASE *V*. A child of three developed an acute otitis media following an upper respiratory infection (acute coryza). The ear was incised and drained freely for several days, when it suddenly ceased to discharge. The following morning the child had fever, vomited, and had a typical scarlatinal rash, without angina. The ear was reopened, and the rash and symptoms immediately subsided. The Dick reaction on the following day was questionably positive. Cultures from the ear showed a scarlatinal streptococcus as judged by toxin production. In this instance there was probably almost complete immunity to the toxin, yet when confined there was sufficient toxin in the discharge from the ear to cause a rash.

CASE *VI*. A child of six developed scarlet fever in May. She had been in contact with no other children except her brothers and sisters for the previous week. Scarlet fever was not prevalent. The mother had had an acute antrum two weeks previously. When the child was taken ill this antrum infection had subsided, but cultures from the nose showed streptococci which produced scarlatinal toxin. The mother had apparently had a bacterial infection of the nose with *Streptococcus scarlatinæ* and was the source of the daughter's infection. As far as she knew she had had no rash.

2970

Roentgen ray therapy in rheumatic heart disease.

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In the course of rheumatic fever, cardiac involvement is frequent and constitutes the chief menace of the disease. No method is known whereby affection of the heart may be effectually prevented. Nor has it been possible significantly to modify the progressive character of the lesions initiated in the structures of the heart by the rheumatic process. These lesions are of two kinds: (1) exudative, representing the reaction to acute infection or intoxication and appearing characteristically in the myocardium as Aschoff bodies; (2) proliferative, the latter denoting attempts at healing, with the formation of scar tissue.

There are reasons for believing that roentgen rays, in suitable doses, might be expected to exercise an effect upon rheumatic lesions in the heart. Radiotherapy has been successfully employed in the treatment of certain low-grade infections and is now standard practice in the management of tuberculous lymphadenitis and acne. Even more acute inflammatory conditions, such as carbuncles and furuncles, are usually favorably influenced. The result of the treatment appears to be an increase in the local resistance to the invading organisms, but the mechanism by which this is brought about is not understood.

Another well-known effect of roentgen therapy is its action upon scar tissue. Cicatrices of the skin become softer and more flexible after irradiation and keloids may be greatly reduced in size. Hence, the possibility was considered that the physical condition of thickened, stiff valve leaflets might be altered. However, the dose necessary to modify cicatricial tissue is greater than that used in the treatment of infections and the time required for the change to appear is much longer. It is probable that this effect played no rôle in the results now reported. It is a problem which we propose to investigate.

In presenting this preliminary report, it is realized that the

number of observations is small. The findings are described without any attempt at their interpretation at this time.

Six cases of rheumatic heart disease have received radiotherapy, two of them having been followed for 9 months. In addition to noting changes in clinical condition, frequent electrocardiograms and numerous teleroentgenograms have been made. The radiation was given over the precordium, the size of the field depending upon the size of the heart. It was usually 15x15 cm. The formula was as follows: 200 K. V. peak; 50 cm. target skin distance; 4 ma.; 0.5 mm. copper and 2.0 mm. aluminum filter. The thickness of the left side of the chest was measured with a pelvimeter and the dose determined with the aid of anatomical and isodosis charts. It was calculated to yield about 10 per cent of the theoretical erythema dose in the region of the mitral valve. This dosage is relatively small, but it was necessary to avoid any possibility of cardiac injury.

In three cases, definite and marked clinical improvement has been observed coincident with changes in the form of the electrocardiogram. In one patient, a girl of 13, with large heart and mitral disease, all the usual therapeutic procedures, including several transfusions, were unavailing during a 9 months hospital stay. Fever and tachycardia continued, although there were negligible joint manifestations. The association of betterment with radiotherapy seemed inescapable. A boy of 16 with mitral and aortic disease and greatly enlarged heart had been bed-ridden for many months because of severe attacks of precordial pain accompanied by a rise in blood pressure, resembling the so-called "angina" of more advanced years. Suffering was so intense that he was transferred from another hospital for cervical sympathectomy. Again, radiotherapy proved strikingly effective, but only after 3 treatments had been given. In a third patient whose progress has been unusually satisfactory, the relationship to radiotherapy is less definite. But the electrocardiographic changes afford presumptive evidence that the lesions in the myocardium have been influenced. Three of the cases have thus far received insufficient treatment to warrant a report.

The alterations in the form of the electrocardiograms following radiotherapy have been of three main types: (1) alteration in the form and direction of the T waves; (2) change in the form, direction and notching of Q. R. S.; (3) change in the duration of P-R time, when this was prolonged. In two instances

occasional ventricular premature beats appeared following exposure to the rays. The irregularity was transitory but was regarded as noteworthy in that it was not observed in many other records. No other disturbances in rhythm were noted.

The form changes are not to be regarded as specific but rather as denoting alteration in the course of the excitation wave through the tissues of the heart. The significant fact is their direct relationship to radiotherapy and their association with clinical improvement. Changes of a similar order are frequently observed in rheumatic fever. Cohn and Swift, who have described them in detail, believe that they are indicative of myocardial involvement. The modification of the form of the electrocardiogram apparently associated with roentgen ray therapy suggests that an influence, presumably favorable, has been brought to bear upon the lesions in the heart muscle.

A limited number of roentgen ray treatments has been given to two patients with *Streptococcus viridans* endocarditis without evident effect either on the course of the disease or on the electrocardiogram.

2971

Changes in biological value of cereal proteins due to heat treatment.

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(Introduced by Carl L. A. Schmidt).

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Young rats taken at weaning were fed diets constituted as follows:

Cereal, 95 per cent.
Salt mixture, 3 per cent (Osborne & Mendel).
Agar, 2 per cent.
Cod liver oil, 25 mg. daily fed separately.
Dry yeast, 0.5 gram daily fed separately.

This mixture should support normal growth provided the

intake is sufficient and the quantity and quality of proteins contained in the cereal are adequate. The cereals thus tested were, (1) raw cracked whole wheat, (2) cracked whole wheat cooked with excess of water, (3) cracked whole wheat toasted, (4) baker's white bread, (5) baker's white bread toasted in ¼-inch slices, (6) crumb of white bread, (7) crust of white bread, (8) puffed wheat, (9) shredded wheat, (10) cream of wheat, (11) puffed rice, (12) raw rice.

From four to twelve animals were kept from eight to sixteen weeks on each of these diets, with accurate food intake records. Practically normal growth was obtained upon the raw wheat, water-cooked wheat, white bread, crumb of bread, shredded wheat, cream of wheat. Raw rice gave rather less than normal growth. Very much retarded growth was obtained upon the puffed wheat, puffed rice, toasted wheat, toasted bread and crust diets. There was some variation in food intake, but growth calculated in terms of gain per gram of food, or of protein eaten, indicates definite inferiority of the latter diets.

For example, the average gains in grams of body weight per gram of protein eaten during the eight weeks of rapid growth, when the animals were four to twelve weeks of age, were as follows: toast, 1.3; bread, 1.5; cracked wheat, 1.8; puffed wheat, 0.9. No serious differences in protein content exist among these preparations. These figures are quite similar to those obtained by Osborne and Mendel[1] in their comparable study of the nutritive value of cereal proteins.

In order to test the theory that protein changes due to heat treatment may account for these differences, a similar number of animals were fed these same twelve diets with replacements of five per cent of cereal with purified casein. In all cases normal growth resulted. The comparable figures for the four diets mentioned above with five per cent of casein included are: toast, 2.0; bread, 2.0; cracked wheat, 1.8; puffed wheat, 1.8.

Digestibility experiments were carried out upon twenty-four of these animals in three periods of five days each in order to determine whether losses in digestion account for the differences. Although some variation was found, it was clear that this factor alone cannot account for the differences.

Comparisons of the biological value of the proteins by the

[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 275.

modified Thomas[2] procedure proposed by Mitchell[3] are now under way. Similar feeding experiments with isolated cereal proteins which have been heat treated, and with water-extracted cereals to eliminate the effect of possible caramel-like bodies are also under way.

2972

The nature of immunity to a protozoan infection.

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In malaria infections it is a common observation that some people are more resistant than others, an observation confirmed experimentally by Celli in 1901[1] and more recently by Yorke and Macfie,[2] who showed that there are some individuals who are resistant to experimental infection.

An experimental study of this problem with malaria parasites was out of the question until the advent of the treatment of paresis by a superimposed malaria infection. We, therefore, decided to study the problem in the case of another protozoan infection, the trypanosome, which produced in experimental animals a relapsing, fatal, disease.

Our studies thus far have brought out a number of facts bearing on this problem:

(1) *Acquired immunity after cure.* None of the animals, rabbits or guinea pigs, used in our experiments, and the number is now fairly large, failed to become infected, or recovered spontaneously from the infection. However, animals cured of the infection with Bayer 205 acquire a resistance to reinfection quite distinct from the possible protective action due to the drug itself. These experiments have been repeated many times, with both rabbits and guinea pigs and the result is quite constant. Infected

[2] Thomas, K., *Arch. Physiol.*, 1909, 219.

[3] Mitchell, H. H., *J. Biol. Chem.*, 1924, lviii, 873.

[1] Celli, A., *Die Malaria*, 1913.

[2] Yorke, W., and Macfie, T. W. S., *Trans. Soc. Trop. Med. and Hyg.*, 1924, xviii, 13.

animals treated and cured with minimal amounts of Bayer 205 (0.05 gm. per kilo), which in control animals gave only temporary protection,[3] became refractory to repeated attempts at reinfection for at least three to five months. Kleine and Fisher,[4] working with monkeys, also found that the cured animals were more resistant to reinfection than normal animals receiving the same dose of the drug.

This is, therefore, an experimental demonstration of an acquired immunity to a protozoan infection. Similar results have recently been reported by Chesney and Kemp,[5] and by Voeigtlin and Dyer,[6] in experimental treponema infections in rabbits cured with Salvarsan or other drugs. Apparently animals cured from a protozoan infection manifest an acquired resistance to reinfection.

These results opened two lines of investigation. On the one hand we studied the possibility of artificial immunization, and on the other we tried to ascertain the mechanism of the acquired resistance.

Artificial immunization to trypanosome infection. Attempts to immunize rabbits against trypanosomes by the usual methods of repeated infection of the organisms yielded most unexpected results. The trypanosome material was obtained from heavily infected guinea pigs, by centrifugation and washing. Whatever method we employed we obtained increased sensitization to infection instead of increased resistance. In one series of experiments two sets of rabbits were treated with disintegrated trypanosomes, obtained by repeated freezing and thawing. The animals received three injections at intervals of five days. Other sets were inoculated over long periods (ten, twenty and twenty-five injections), with whole trypanosomes. But whatever the method of treatment, the animals became so sensitive that the incubation period after the infection was about one fourth or less than the usual interval. The blood infection during the first few days was heavy and many of the animals showed swelling of the eyelids and ears, with the onset of the infection.

Since the immunity observed in cured animals developed after treatment with a drug (Bayer 205), we repeated the immuniza-

[3] Kligler, I. J., and Weizman, I,. *Ann. Trop. Med.*, 1924, xviii, 437.

[4] Kleine, K. F., and Fisher, W., *Deutch. Med. Wchnschr*, 1922, xlviii, 1693.

[5] Chesney and Kemp, *J. Exp. Med.*, 1924, xxxix, 553.

[6] Voegtlin, C., and Dyer, H. A., *Pub. Health Rep.*, 1925, xl, 2511.

tion experiments, using mixtures of Bayer 205 and trypanosomes. The trypanosomes were obtained from infected guinea pigs, as before, and mixed with amounts of the drug (0.005 gm. per kilo), known from previous experiments not to give any protection against infection.

This procedure gave totally different results than those reported above. The animals treated with the mixture developed a degree of resistance, in all of the experiments, quite distinct from that conferred by the drug alone. In one experiment the treated animal never developed the infection, and in the others the incubation period was greatly prolonged, usually about twice as long as that in the control animals treated with the drug alone. Why the injection of trypanosomes alone sensitizes the animals while trypanosomes mixed with Bayer 205 increases their resistance is a problem for further investigation.

Mechanism of resistance. The determination of the nature of the resistance presented certain difficulties. The species of trypanosome used in the experiments, *Tr. evansi,* produces a chronic, relapsing infection which is always fatal. This implies that there is a native resistance slowly broken down by the parasite. It seemed likely that the acquired and native resistances are in some way related, but their nature was not clear. Attempts to identify humoral antibodies *in vitro* tests, and by the Pfeifer phenomenon in guinea pigs, gave negative results. Recently Luengo and de Buen[7] claimed to have demonstrated specific lytic substances in the blood serum of a patient cured with Bayer 205, using the mouse as the test animal. These results were confirmed by Kiang[8] in experimental animals, also using mice as the test animals.

Our experiments indicate that the natural and acquired resistances are closely related to the form elements of the blood. Infected animals treated with large doses of olive oil (5 to 10 cc.) relapse very promptly, usually in 24 to 48 hours after the injection. Similarly cured animals, receiving an injection of a large dose of oil followed by an infective dose of trypanosomes, develop an infection, when other animals cured at the same time and reinfected with the same dose still resist the infection. Finally, animals in which the leucocytes have been destroyed by the in-

[7] Luengo, E., and De Buen, S., *C. R. Soc. Biol.*, 1924, xci, 825.

[8] Kiang, T. S., *Arch. f. Schiffs u. Tropen. Hyg.*, 1925, xxix, 572.

jection of benzol develop a blood infection after an incubation period of one day, or about one fourth to one sixth the usual time.

These experiments indicate that the mechanism of resistance resides to a large extent, if not wholly, in the form elements of the blood, or, as it is usually designated, the reticulo-endothelial system. Both the oil and the benzol have some effect on the large endothelial cells. Olive oil apparently renders them ineffective by blocking them. An examination of the peritoneal fluid during the first few days after its injection shows a very large increase in the large mononuclear cells, in a state of great activity, many of them filled with the oil. It is probably this blocking that results in a lowered resistance of the animal to the invasion of the parasite. After recovery, a week or so after the injection, the resistance of the animal is apt to be greatly increased. Benzol produces the same effect in a different manner. Injection of benzol causes a primary leucocytosis, followed by a rapid destruction of the leucocytes. During this period the resistance of the animal is greatly decreased, so that the multiplication of the parasites proceeds rapidly and the blood is promptly invaded. On recovery the resistance of these animals may be even greater than that of normal animals, and blood invasion is more uncommon than in normal controls.

Summary. These experiments show: (1) That trypanosome infected animals cured with Bayer 205 develop acquired resistance to reinfection lasting several months. (2) That repeated injections of trypanosomes causes hypersensitization. (3) That injection of a mixture of trypanosomes and Bayer 205, leads to partial immunization. (4) That the resistance, both natural and acquired is due in large measure to endothelial cells, because blocking with olive oil or destruction of the leucocytes with benzol breaks down the resistance.

2973

Can taurine replace cystine in the diet of the young white rat?

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Mitchell[1] has reported that taurine and cystine were equally efficient in promoting growth in white mice on a diet (source of protein, casein) in which cystine was the limiting factor. In the present series of experiments, young white rats have been maintained on the milk powder-starch diet of Sherman,[2] supplemented by vitamin B yeast concentrate tablets. On this basal diet slow growth was possible. When the basal diet was supplemented with cystine (0.3 per cent) marked increase in the rate of growth was obtained. When taurine (prepared from ox bile) was used as a supplement (1 per cent), no increase in the rate of growth as compared with the controls on the basic diet could be noted. Typical results on two litter units follow in the table. From our data no evidence is to be obtained in support of the contention that the young white rat can use taurine for the purposes of growth in the absence of sufficient cystine.

Litter No.	Duration Weeks	Diet.	Number of rats	Initial weight	Final weight	Gain
				gm.	gm.	gm.
1	10	Basal	3	57.0	120.5	63.5
				55.0	109.5	54.5
				47.0	95.0	48.0
	10	Basal + Taurine	3	50.5	95.5	45.0
				50.5	91.0	40.5
				48.0	99.5	51.5
	10	Basal + Cystine	3	47.0	132.0	85.0
				41.0	112.5	71.5
				47.5	141.5	94.0
2	6	Basal	2	84.0	109.0	25.0
				85.0	114.5	29.5
	6	Basal + Taurine	2	84.5	112.5	28.0
				88.5	113.0	24.5
	6	Basal + Cystine	2	79.0	129.0	50.0
				54.0	106.0	52.0

[1] Mitchell, M. L., *Austrail. J. Exp. Biol. Med. Sci.*, 1924, i, 5.

[2] Sherman, H. C., and Merrill, A. T., *J. Biol. Chem.*, 1925, lxiii, 331.

2974

A continuous method of studying hydrogen ion concentration of urine during secretion.

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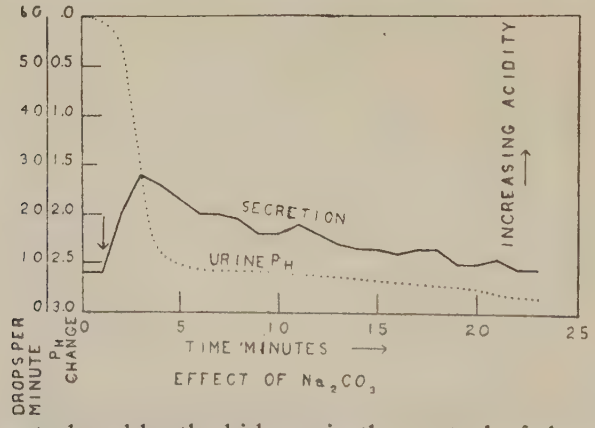
The continuous method of recording the hydrogen ion concentration of the circulating arterial and venous blood[1] has been applied to the urine flowing through the ureter from the kidney. The purpose of the work is to study the mechanism of renal secretion, the relation of the cH of the urine to the cH of the blood and the cerebro-spinal fluid, in the hope of elucidating the mechanism of respiratory control. The application of the method to urine promises to be of value. The administration of various salts such as sodium carbonate, ammonium chloride, and magnesium sulphate, gave characteristic curves of changes in hydrogen ion concentration. The accompanying graph shows the im-



portant part played by the kidneys in the control of the acid-base equilibrium of the body. The large and prolonged increase in secretion and in alkalinity of the urine agrees with the recovery of the blood towards normal reaction after a similar injection of sodium carbonate.

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[1] Gesell, Robert, and Hertzman, Alrick B., PROC. SOC. EXP. BIOL. AND MED., 1925, xxii, 298.

2975

The effect of ligation of the hepatic artery on carbohydrate metabolism.*

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In the course of some experiments on the physiology of the liver of the dog, we observed that ligation of the hepatic artery caused death of the animals in convulsions. Previous investigators[1, 2, 3] observed death in convulsions in animals after complete circulatory exclusion of the liver. Mann and Magath[4] found that after complete hepatectomy their dogs died in hypoglycemic convulsions. We, therefore, undertook the study of the relation of the blood sugar level to the convulsive seizure following ligation of the hepatic artery.

There was a remarkable constancy in the results obtained. After coming out of the anesthetic, the dogs appeared normal—walked, ran, barked, drank water and excreted normal amounts of urine and feces. The dogs remained in this apparently normal state from 15 to 50 hours, following which they became drowsy, listless, and unresponsive. Pinching or pricking did not call forth any response. The animals refused food and water. During this stage occasional fibrillary twitches were observed. Then (within 1 to 2 hours) they manifested symptoms of hyperirritability, hyper-reflexia and a sudden onset of generalized clonic convulsions followed by tonic spasms, retraction of the neck, respiratory paralysis, an occasional sharp yelp, and death. This general seizure usually lasted from 2 to 5 minutes.

* With the aid of a grant from the Therapeutic Research Committee of the American Medical Association.

1 Hahn, M., Massen, O., Nencki, M., and Pawlow, J., *Arch. für Exper. Path. u. Pharmakol.*, 1893, xxxii, 161.

2 Matthews, S. A., and Miller, E. M., *J. Biol. Chem.*, 1913, xv, 87.

3 Whipple, G. H., and Hooper, C. W., *J. Exper. Med.*, 1913, xvii, 593; *Ibid.*, 612.

4 Mann, F. C., and Magath, T. B., *Arch. Int. Med.*, 1922, xxx, 73; *Ibid.*, xxx, 171.

Coincident with these observations, the blood sugar curve was studied. It was found that there was a definite parallelism between the blood sugar level and the symptoms just described. When the blood sugar was within normal limits, the animals did did not manifest any symptoms, but as soon as it has dropped to 60 mg. per 100 cc. of blood or below, the dogs developed the first stage of the syndrome, that of drowsiness. The blood sugar dropped progressively and when it reached a level below 45 mg. per 100 cc. of blood, convulsions and death supervened.

Immediately after death autopsies were performed, and portions of the liver, skeletal and heart muscle were at once examined for glycogen. In no case were we able to find glycogen in these tissues either by alcohol precipitation or positive reduction after hydrolysis. Microscopic studies of the liver were made, the histo-pathology of which is being studied at present, but the results are not mature to be discussed at this presentation.

In a few of our animals the administration of glucose orally or intravenously was successful in bringing the animals out of convulsions and prolonging the period of survival for several hours.

To eliminate the possibility of the picture being produced by nerve injury, the plexus of nerves surrounding the hepatic artery was severed in one dog without ligating the hepatic artery. This procedure in no way interfered with the well being of the animal. The dog is still alive and normal since the operation on December 31, 1925.

CONCLUSIONS.

(1) Ligation of hepatic artery of dogs causes death in hypoglycemic convulsions.

(2) The period of survival after ligation depends upon the amount of glycogen previously stored, and varies between 15 to 60 hours.

(3) There is a total depletion of glycogen in the tissues at death.

(4) Administration of glucose prevents convulsions and prolongs life for several hours.

(5) Severing the plexus of nerves surrounding the hepatic artery does not produce this hypoglycemic syndrome.

2976

Anesthesia induced by barbituric acid derivatives with special reference to associated blood sugar changes.

SOMA WEISS.

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During an attempt to study the regulatory influence of the central nervous system on the carbohydrate metabolism, it was noted that cats under the effect of iso-amyl-ethyl barbituric acid (amytal) developed hyperglycemia.

It is recognized that narcosis, however induced, is associated with changes in the blood sugar. Whether this change is due to specific drug effect, or whether it is a part of physiological changes associated with narcosis is an open question. Page's[1] observation that anesthesia induced by iso-amyl-ethyl barbituric acid (amytal) is not associated with blood sugar changes in rabbits and dogs is, therefore, of interest, though Bang[2] found that diethylbarbituric acid (veronal) raises the blood sugar of the rabbit, and Ellis and Barlow[3] state that the blood sugar of pigeons and cats is lowered several hours after the intraperitoneal injection of the sodium salt of diethylbarbituric acid (medinal).

In numerous investigations during the past years, various derivatives of barbituric acid, especially iso-amyl-ethyl barbituric acid (amytal) have been used for anesthesia, and from the blood sugar changes far reaching conclusions have been drawn as to changes in carbohydrate metabolism.

The investigation to be reported consists of fifty-three experiments on thirty-two cats and on five dogs. The freshly prepared sodium salts of diethybarbituric acid (in doses of 120, 150, 180, and 300 mg. per kilogram of body weight), of iso-amyl-ethyl barbituric acid (doses 38, 50, 60, 65, 80 mg. per kg. b. w.), and of iso-allyl-propyl barbituric acid (50, 60, 80 mg. per kg. b. w.) were injected intraperitoneally and intravenously. Blood sugar

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[1] Page, I. H., *Lab. and Clin. Med.*, 1923, ix, 194.

[2] Bang, I., *Biochem. Ztschr.*, 1913, lviii, 236.

[3] Ellis, N. N., and Barlow, O. W., *J. Pharmacol. and Exp. Therap.*, 1924, xxiv, 259.

determinations were made according to Benedict's[4] "1925" method. After the blood sugar of the fasting animals was determined, repeated blood samples were taken up to six to twenty-four hours respectively following the administration of the hypnotics. The hemoglobin content was determined according to Cohen and Smith.[5] Blood was obtained from the jugular or femoral veins. The veins were exposed through a small nick, made with a sharp knife under phenol oil, local anesthesia. The animals were handled gently, and that no excitement was produced, is shown by the fact that the average blood sugar of the cats after having been prepared for experiment was 95 mg., that of the dogs 96 mg.

It may be stated, without going into the details of the experiment, that the administration of the three derivatives of the barbituric acid tested was associated with changes in the blood sugar. The change was more marked in the cat. All the blood sugar curves, following the administration of the hypnotics, show a sudden rise, reaching a maximal height within one or two hours after the injection. The increase may be 100 to 250 per cent of the normal value in cats and 80 to 150 per cent in dogs. The sudden initial rise of the curve is followed by a gradual fall, lasting as long as six to twenty hours, in the majority of the experiments. In the small number of the experiments there was a plateau, or a further very slow rise over four to six hours after the initial peak. In two dogs after an initial rise, the blood sugar fell below the normal value.

It is well known that the sympathetic nervous system of the cat is sensitive and active. In order to rule out the possiblity that excitement due to handling of the animal might have precipitated a late rise in the blood sugar, four cats were injected intraperitoneally with iso-amyl-ethyl barbituric acid, while in their cages and then left there until they were under the effect of the drug (thirty to seventy minutes). The blood sugar changes in these animals corresponded to the figures obtained on other animals, in which precaution was not taken.

Three cats were tied on the board without anesthesia and after the normal specimens were obtained, they were kept there for ten to thirty minutes in order to produce marked excitement. They

[4] Benedict, S. R., *J. Biol. Chem.*, 1925, lxiv, 207.

[5] Cohen, B., and Smith, A. H., *J. Biol. Chem.*, 1919, xxxix, 489.

were then returned to the semi-dark cage room. One and one-half hours later, the blood sugars were found normal. These experiments indicate that if excitement produces rise in the blood sugar, the latter falls abruptly to the normal value soon after the animals are in a quiet state. The result indicated that the duration of the changes in the blood sugar due to stimulation of the sympathetic nervous system through excitement depends on the duration of the stimulus and that, therefore, late changes in the blood sugar can not be attributed to an early short excitement.

It was thought that the initial rise in the blood sugar might be due to the stage of excitement of the lower sympathetic brain centers during the early stage of anesthesia. In order to shorten this period as far as possible, the hypnotics were injected intravenously. The blood sugar curves of these animals did not, however, differ from those obtained after intraperitoneal administration.

To compare the effect of sympathetic stimulation due to excitement of the blood sugar with that after the administration of the hypnotic, the blood sugar curves of four cats, kept on the board without anesthesia on the first day of the experiment, were compared with that determined on the second day after the intraperitoneal administration of iso-amyl-ethyl barbituric acid. Experiments of the reversed order were performed on three cats. The maximal rise of the blood sugar was always reached sooner in the experiments with hypnotics. The intensity of the rise was more marked on the first day of the experiment, suggesting that the animals cannot respond on the second day with such marked hyperglycemia, because their glycogen stores are partly exhausted.

The investigation suggests that the blood sugar changes, under experimental conditions described are due, at least partly, to the action of the barbituric acid derivatives. The pharmacodynamic action of this group of drugs on centers in, and perhaps around, the thalamus would suggest that the disturbance of these centers may be responsible for blood sugar changes. One observes, not infrequently, that pathological changes of these centers in man are associated with temporary disturbance of the carbohydrate metabolism.

One cannot say whether these changes are due to stimulation or depression of the centers. It is of interest that ergotoxin phosphate, when injected intravenously into cats, produces sym-

pathetic depression within 2 to 6 minutes, nevertheless, the animals showed blood sugar changes similar to that observed after the administration of the barbituric acid derivatives.

Changes in the hemoglobin content of the blood were not sufficiently uniform to justify conclusions as to changes in the blood concentration during the period of anesthesia.

Animals injected with the same dose of the identical solution of the hypnotics showed marked variation in the depth, in the uniformity of the narcosis, and in the severity of the disturbance in the heat regulation.

From these, and previously performed, and hitherto unpublished experiments on cats and dogs, there seems to be no justification for concluding that certain derivatives of barbituric acid are superior to others, as to safety and uniformity of sleep. This conception is substantiated by the numerous contradictions in the literature on the subject. The stage of excitement was perhaps longer and more marked in dogs after diethyl barbituric acid, than after the other derivatives tested.

When dilute solutions of the hypnotics were injected from a burette intravenously for a period of three hours, as large amounts as 166 mg. of amytal and 360 mg. of veronal per kilogram of body weight of cat were not fatal. Even by rapid intravenous administration the toxicity of the three derivatives is not more, but perhaps less, than after intraperitoneal or subcutaneous administration. Animals occasionally ceased to breathe rather abruptly during the stage of excitement. These findings illustrate the difficulties to be encountered in the determinations of the toxicities of the barbituric acid derivatives by intravenous administration.

After this investigation was completed, Hines, Boyd, and Leese,[6] in studying the response of dogs to intravenous glucose administration, report that certain phases of the carbohydrate metabolism are disturbed under the iso-amyl-ethyl barbituric acid anesthesia.

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[6] Hines, H. M., and Boyd, J. D., and Leese, C. E., PROC. SOC. EXP. BIOL. AND MED., 1925, xxiii, 228.

Missouri Branch.

Washington University School of Medicine, January 20, 1926.

2977

Quantitative experimental data on the sympathetic innervation and tonus of triceps brachii muscles.

ALBERT KUNTZ and ALVER H. KERPER.

[*From St. Louis University School of Medicine, St. Louis, Mo.*]

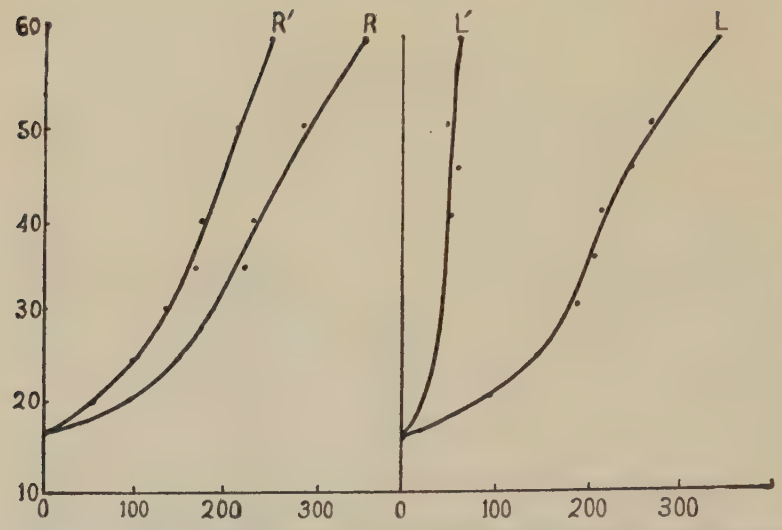
In a recent paper[1] the writers reported briefly the results of tonus measurements on the quadriceps femoris muscles in cats and dogs, obtained by the use of Spiegel's method,[2] before and after elimination of the sympathetic nerve supply to these muscles, and before and after removal of the cerebellum. By the use of the same method, tonus measurements of the triceps brachii muscles in cats and dogs were obtained before and after elimination of their sympathetic, and before and after elimination of their somatic nerve supply.

The tonus curves, *i. e.*, the curves of passive extension, of the triceps brachii, obtained under comparable conditions, are similar to those of the quadriceps femoris. However, the components of tonus mediated through the sympathetic and the somatic innervation, respectively, can be studied more advantageously in the triceps than in the quadriceps, by reason of the fact that either the sympathetic or the somatic nerve supply to the former may be cut off, leaving the other intact. In the case of the quadriceps the sympathetic supply may be cut off without impairing the somatic, but the somatic supply may not be cut off without impairing the sympathetic. In our experiments the sympathetic nerve supply to the triceps was elminated by extirpation of the inferior cervical sympathetic ganglion. The somatic supply was cut off

[1] Kuntz, A., and Kerper, A. H., PROC. SOC. EXP. BIOL. AND MED., 1925, xxiii, 77.

[2] Spiegel, E. A., *Z. f. d. ges. Neur. u. Psych.*, 1923, lxxii, 517.

FIGURE 1.



R. and L., tonus curves of the normal right and left triceps brachii muscles respectively of a dog. R′, tonus curve of the right triceps brachii muscle following division of both roots of the sixth, seventh and eighth cervical nerves. L′, tonus curve of the left triceps brachii muscle following extirpation of the left inferior cervical sympathetic ganglion.

by division of both roots of the sixth, seventh and eighth cervical nerves within the vertebral canal.

If the limb is only slightly flexed at the beginning of measurement, the tonus curve of the normal triceps (Fig. 1, R. L), like that of the normal quadriceps, rises very slowly at the beginning and then more rapidly as passive flexion of the limb increases. The greater resistance offered by the muscle at the beginning of passive extension, indicated by the slow rise in the curve, constitutes the so called "brake phenomenon". Following elimination of the somatic nerve supply, leaving the sympathetic supply intact, the resistance of the muscle to passive extension is diminished somewhat, but the brake phenomenon is still exhibited. This is illustrated by curve R′ in the figure. This curve represents the component of tonus which is mediated through the sympathetic innervation. Following elimination of the sympathetic nerve supply, leaving the somatic supply intact, the resistance of the muscle to passive extension is diminished to a far greater degree and the brake phenomenon is absent. The tonus curve (Fig. 1,

L') rises rapidly from the beginning. This curve represents the component of tonus which, in the absence of contractile effort, is mediated through the somatic nerves.

The results of these experiments corroborate the results of the tonus measurements on the quadriceps femoris muscles, reported in the previous communication.[1] Furthermore, they demonstrate the existence of tonus in a skeletal muscle in the absence of functional somatic nerves. Doubtless this component of tonus is plastic in quality. Therefore, additional evidence, both quantitative and positive in character, is afforded in support of the theory that plastic tonus in skeletal muscles is mediated through the sympathetic nervous system.

2978

Conditions affecting the formation of glycuronic acid in rabbits.

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[*From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Mo.*]

This experiment represents preliminary data of a study, the object of which is to determine the factors which affect the formation of glycuronic acid in the body.

A solution of menthol was made by dissolving 44 grams in enough warm olive oil to make a total volume of 66 cc. Six cc. portions were administered to rabbits through a stomach tube by means of a graduate syringe. Only healthy young animals were used.

Date	No. of animals group	Av. wgt. in kilos	Treatment	Gm. menthol per rabbit	Excretion of crude ammonium menthol glycuronate per rabbit
7/ 8/25	6	2.04	Normal: food + glucose	4.0	4.0
7/11/25	5	2.00	Insulin: food + glucose	4.0	2.6
7/14/25	4	—	Normal: food	4.0	3.7
7/16/25	4	1.95	Phlorhizin: starving	4.0	2.2

The results indicate clearly that the glycuronic acid output may be decreased either by insulin or by phlorhizin. The normal output per rabbit after the above dose of menthol was about 4 grams of the crude ammonium menthol glycuronate. Under the same conditions, but with insulin and glucose, the output was only about 2.6 grams per rabbit. When starved and phlorhizinized, similar results were obtained, as in the case of insulin. This appears to indicate a relation between the amount of carbohydrate in the tissue and the extent of synthesis of glycuronate.

2979

Ketolytic action of various sugars in vitro.

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The results here summarized represent a continuation of the study of the *in vitro* reactions between sugars and acetoacetic acid discovered in 1921 by Shaffer[1] and are believed to afford an explanation of antiketogenesis.[2]

The reactions of aldehydes and sugars were studied under two conditions: (1) "non-oxidative" and (2) "oxidative".

"NON-OXIDATIVE REACTION."

Simple aldehydes such as formaldehyde,[3] acetaldehyde, glyoxylic acid, glyoxal,[4] and glyoxal carbonic acid reacted readily with potassium acetoacetate at pH 8.0 (in the absence of any oxidizing agent). *Under the same conditions only simple aldoses reacted.* Thus glycol aldehyde and glyceric aldehyde reacted with acetoacetate. *Dihydroxy acetone* did not react and in this respect it acted like other ketones such as pyruvic acid and acetone. How-

[1] Shaffer, P. A., *J. Biol. Chem.*, 1921, xlvii, 433.

[2] Shaffer, P. A., *J. Biol. Chem.*, 1921, xlvii, 449.

[3] Shaffer, P. A., "Antiketogenesis: Its Mechanism and Significance." *Harvey Society Lectures*, 1923-1924. Lippincott Company, Philadelphia.

[4] Shaffer, P. A., and Friedemann, T. E., *J. Biol. Chem.*, 1924, lxi, 585.

ever, when the solution of dihydroxy acetone was made strongly alkaline it reacted as readily as glyceric aldehyde, showing that rearrangement of the inert ketone form are necessary before reaction may occur. The more complex sugars, the pentoses and hexoses, did not react in non-oxidative neutral media, but they became reactive in strongly alkaline solutions,[5] the alkali perhaps opening the ring and producing the free aldehyde form of sugar which latter form is active in a manner similar to glycol- and glycer-aldehydes.

Although these reactions occurred in dilute solution (20 millimols aldehyde : 80 millimols acetoacetate per liter) they were more rapid and extensive in concentrated solution. In concentrated solutions the total acetoacetate consumption approached two molecules for each molecule of added aldehyde, never greater than two. In the case of acetaldehyde, where it was possible to determine both aldehyde and acetoacetate consumption, a similar 1 to 2 ratio was found. This again shows the similarity to the Knovenagel reaction.[4]

TABLE I.

33.4 mMols. Acetaldehyde + 102.3 mMols. K acetoacetate per liter. 23 hrs. in water bath at 37.5° C.

	millimols
Acetaldehyde consumed	15.4
Acetoacetate consumed	28.3
CO_2 liberated as $KHCO_3$	32.1

"OXIDATIVE REACTION."

Twenty millimols of aldehyde or sugar were oxidized by 200 millimols H_2O_2 in 0.5 N KOH in the presence of 80 millimols potassium acetoacetate. *It was found that only those sugars which reduced Fehling's solution were ketolytically active.* Aldehydes, even though some of them (glyoxylic acid and glyoxal carbonic acid) reduced Fehling's solution, were inactive. Dihydroxy acetone, which in non-oxidative solutions was inert, became exceedingly reactive, its reaction rate coinciding with glyceric aldehyde.

The reaction rates for glycol- and glyceric aldehydes, which were very much greater in the oxidative medium, and dihydroxy acetone, did not show an initial lag such as was always found in the case of the pentoses, hexoses, heptoses, and polyoses. This lag, in the case of the sugars possessing ring structures, showed

[5] Friedemann, E. E., *J. Biol Chem., Proc.*, 1925, lxiii, 21.

TABLE II.

In 1000 cc. 37.5° C. { 20 mMols. Sugar; 500 cc. "purest" KOH
75-80 mMols. K Acetoacetate; 200 mMols. H_2O_2

	Acetoacetate consumed by one mol. of sugar*
Diose: Glycol aldehyde	1.9
Triose: Glycer aldehyde	2.2
Dihydroxy Acetone	2.2
Pentose: l-Arabinose	2.7
l-Xylose	2.7
Hexose: d-Glucose	2.4
d-Fructose	2.3
d-Mannose	2.1
Heptose: d-Glucoheptose	2.2
Mannoketoheptose	2.7
Disaccharoses: Maltose	2.0
Lactose	1.8
Sucrose	.2

* These results represent the total consumed in 12 hours minus the blank. Thus 20mMols. dihydroxy acetone consumed 54.6 mMols. acetoacetate, of which 9.9 were calculated to be due to *direct* oxidation. The amount consumed by the sugar was therefore 54.6–9.9, or 44.7.

that an opening of the ring or other transformations are necessary before reaction may occur. This open-ring, reactive form is probably the substance which condenses with aceto acetate, it is believed. That the sugar molecule as a whole, and not a fragment, participates in the condensation, is the only conclusion one may draw from the data shown in Table II.

2980

On conduction of the action potential wave through the dorsal root ganglion.

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It has been reported[1] that the action potential wave started by stimulation of the sciatic nerve arrives in the dorsal roots of that nerve somewhat later than in its ventral roots. Further experi-

[1] Erlanger, J., Bishop, G. H., and Gasser, H. S., *Am. J. Physiol.*, 1925, lxxii, 197.

mentation now makes possible the presentation of additional data bearing on this observation. In fifteen quantitative determinations in the bullfrog with the cathode ray oscillograph, the delay of the quickest sensory behind the motor potential wave ranges, with but one exception (in which, due probably to damage to the ventral root, the value was 0.02σ), between 0.09 and 0.18σ. Omitting the exception, the average delay is 0.143σ. Six determinations in the green frog range between 0.04 and 0.14σ, with an average of 0.08σ. One determination in the cat showed a delay of 0.08σ (temp. 35° C.).

We have been able to show that the delay is not due to differences in the rate of propagation of the action potential wave in sensory and motor fibers, either in the nerve trunk or in its roots. Indeed, the fastest sensory fibers conduct at least as fast as the fastest of the motor fibers. The delay must, therefore, develop in the dorsal root ganglion, but how, or in what division of the part of the neuron within the ganglion, it is at present impossible to say.

2981

The determination of the harmlessness of food colors.

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Fatal doses of certified and other food colors, for cold and warm blooded animals, compared with maximal harmless doses based on the rate of growth of young rabbits, showed no definite relation. It was inferred that harmlessness cannot be derived from fatal doses.

The *food color factor of safety* for man is defined as the quotient of the maximal harmless oral daily dose, divided by the maximal possible daily consumption in foods and beverages by man; the abbreviated formula being $\mathrm{FCFS} = \frac{\mathrm{MHD}}{\mathrm{MC}}$. By the maximal harmless oral dose is meant the largest daily dose, which when given by mouth throughout the life of the most susceptible

laboratory mammal, produces no depression of any physiological process, and no injury to any tissue.

Food colors as a whole are to be regarded as harmless when the total possible daily consumption of all food colors, in all foods and beverages, does not exceed the maximal harmless dose.

A determination of the factor of safety of a food color of medium toxicity, based on the preliminary results for the maximal harmless dose for the growth of young rabbits, and the maximal possible consumption of the color by man in soda water, gave the following result:

$$FCFS = \frac{MHD}{MC} = \frac{21000}{25} = 840.$$

2982

Smooth muscle response in anaphylaxis. I. Effect of mixtures of antigen and sensitized lung tissues.

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Anaphylaxis, according to present conception, is essentially a cellular phenomenon. Of the tissues that are known to participate in anaphylactic shock, that of smooth muscle has received most attention. It has been shown both in the living sensitized animal[1] and also *in vitro* by the Dale experiment[2] that when certain organs containing smooth muscle are exposed to the antigenic substance to which the animal is sensitized, this smooth muscle responds by contraction. Such smooth muscle contraction is responsible for many anaphylactic symptoms. In guinea pigs, with which these experiments are concerned, anaphylactic death is due primarily to the contraction of the highly developed smooth musculature of the bronchioles with subsequent asphyxia. As this may be entirely a peripheral reaction, as Auer[3] has shown,

[1] Manwaring, W. H., Hosepian, R., Enright, J. R., and Porter, Dorothy F., *J. Immun.*, 1925, x, 567.

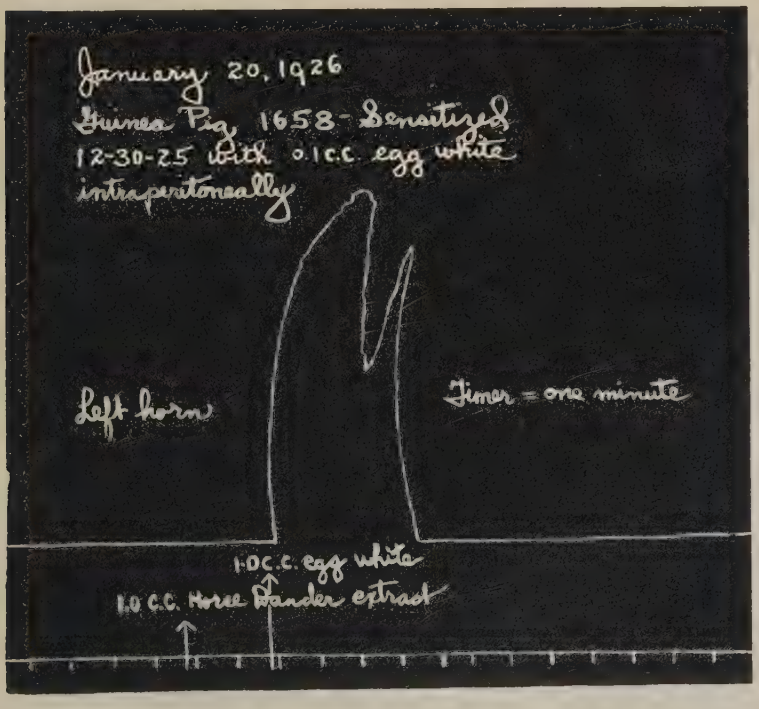
[2] Dale, H. H., *J. Pharm. and Therap.*, 1913, iv, 167.

[3] Auer, J., *J. Exp. Med.*, 1910, xii, 638.

the injected antigen presumably is transported by the blood to the lungs where it finds receptive smooth muscle. Essentially nothing is known, however, about the stimulus which makes smooth muscle contract under these conditions. By utilizing this lung reaction, experiments were attempted to study this problem and the first of these is reported here.

Female virgin guinea pigs of about 250 grams were injected intraperitoneally with 0.1 cc. of egg-white solution with an average nitrogen content of 2.6 mg. per cc.; other lots received 0.5 cc. of horse epithelium extract with a nitrogen content of 0.6 mg. per cc. Both of these were shown to be anaphylactogenic. About twenty-one days later, when optimum sensitivity developed, the animals were killed by fracturing the skull. In each, the uterus was perfused with warm Tyrode solution until free from visible blood. The two anterior uterine horns were then suspended, each in a separate Dale apparatus in which a bath of 250 cc. of

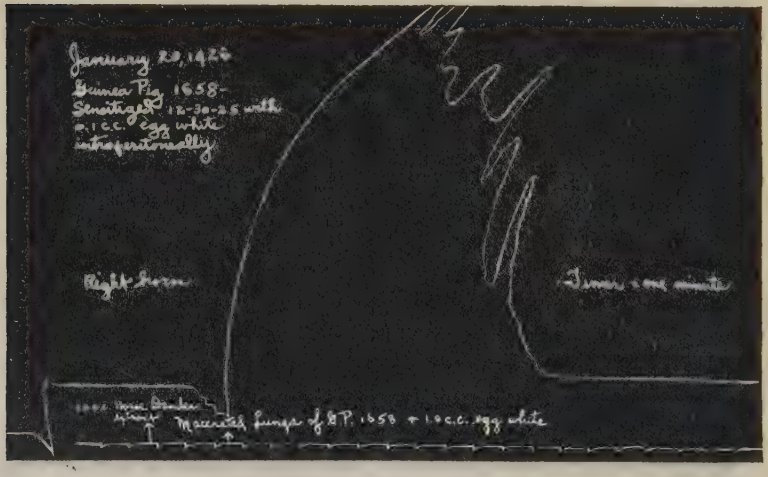
FIGURE 1.



Guinea pig, No. 1658. Left horn. 1.0 cc. of egg white added to Dale bath.

Tyrode solution was used. This was kept at a constant temperature of 37° Centigrade, and a stream of free oxygen was bubbled through it. To one bath containing a suspended horn 1.0 cc. of the homologous antigen was added. In those strips which were sensitive, smooth muscle contraction occurred which was recorded on a kymographic drum and the amplitude of the curve noted (Figure 1). Meanwhile, the lungs from the same animal were dissected from the body and perfused with warm Tyrode solution until as much blood as possible was washed out. The lungs were then ground to a pulp with a mortar and pestle. To this pulp was added the same measured unit of antigen that was used in the Dale bath to detect muscle sensitivity (1.0 cc.) and the mixture ground at room temperature for three minutes. Three cc. of Tyrode solution was added to this mixture to render it partially fluid, and it was then centrifuged at high speed for three minutes. A few cc. of supernatant fluid was drawn off and added to the second uterine horn which, in the meantime, was kept warm and oxygenated in the other Dale bath. To this mixture the suspended muscle strip responded with an amplitude greater than that of the first strip which was exposed to antigen alone (Figure 2.)

FIGURE 2.



Guinea pig, No. 1658. Right horn. Supernatant fluid from mixture of sensitized lung tissue and 1.0 cc. of egg white added to Dale bath.

As controls, ground sensitized lung extract without antigen, when added to a sensitized horn and also when added to a normal horn, caused no contraction in either. Similarly, ground normal lung mixed with antigen when added to a sensitized horn gave a contraction, but not as great a one as compared to that of antigen alone.

When the same experiment was repeated with defibrinated blood and antigen, no exaggerated curves were obtained. On the other hand, liver and uterine tissues both gave curves similar to that of lung tissues.

2983

Type of audiometer for determination of acuity for air and bone transmitted sound.

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A phonograph telephone transmitter is led to the primary of a laboratory induction coil. The secondary of the coil is attached to a loud speaker. A group of individuals may be tested by employing a spoken record, preferably in verse, and gradually shifting the secondary toward the primary until the language is understood. This makes possible a rough separation of the members of the group into those of relatively good and relatively poor acuity. Those with relatively poor acuity may then be re-tested individually by using head-phones instead of the loud speaker; placing the secondary at a remote position and then rotating it across the field of the primary. Or with the secondary in extreme position, a variable resistance may be shunted across the secondary terminals. When the resistance reads zero, nothing passes through the head phones and as the resistance is gradually increased, the threshold may be readily attained and read off in terms of ohms. If the secondary is directly connected to a bone activating telephone receiver such as previously described,[1] the intensity required for bone activation may similarly be empirically determined. The apparatus is suggested as a solution for rapid quantitative testing of school children.

[1] Pohlman, A. G., and Kranz, F. W., PROC. SOC. EXP. BIOL. AND MED., 1923, xxi, 335.

2984

The occurrence and possible functional significance of spiral smooth muscle cells and connective tissue fibers.

JOHN AUER.

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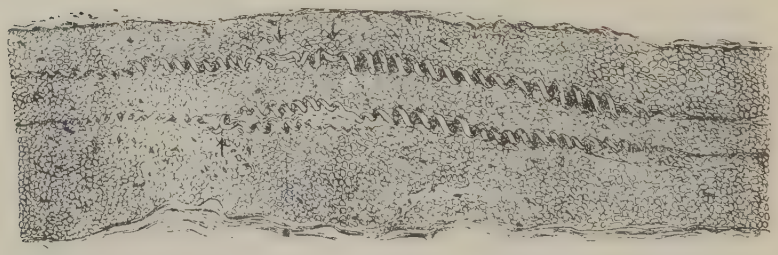
There is a fairly extensive literature on this subject,[1] which cannot be discussed here even though recent work on the contraction of smooth muscle and the production of tonus pays but scant attention to its possible significance. In this place only those findings will be reported, which, as far as I know, have not been described in smooth muscle and connective tissue.

The material studied was the stomach of the frog, colon of the guinea pig, caecum of the rabbit, and duodenum and lower small intestine of the dog. The chief fixative was Orth's solution. The sections were cut from paraffin blocks.[2] The most instructive slides were obtained when the plane of the section was tangential to the surface of the gut or parallel to the direction of the spiral fold in the rabbit's caecum.

Results: Muscle nuclei with a spiral or rather helicoid twist

FIGURE 1.



Section through the spiral caecal fold of a contracted rabbit's caecum. The helicoids are stained a bright red with Van Gieson's stain.

At sites marked by arrows a right twist changes to a left one, or vice versa.

[1] For some of the references see Forster, Edm., *Anat. Anz.*, 1904, xxv, 338; Maresch, R., *Centralbl. f. Allg. Path. u. path. Anat.*, 1905, xvi, 641; Fahr, *Virchow's Arch. f. path. Anat.*, 1906, clxxxv, 29; McGill, C., *Am. J. Anat.*, 1909, ix, 493.

[2] I acknowledge with pleasure that the resources of the Anatomical Department were placed at my disposal by Dr. A. G. Pohlman.

may be observed in every section, particularly if the gut was well contracted. They are most numerous at or near Auerbach's plexus, both in the circular and longitudinal layers. The twist may be a right or a left screwthread, both types occurring in the same field. In numbers of instances the same nucleus showed a right twist in one section and a left twist at the other end. Occasionally the transition site of one twist to the other was beautifully apparent, and on a miniature scale, the same as shown by the accompanying figure 1, observed in white fibrous tissue. The closeness of the twist varied: in the pale vesicular nuclei there was either no twist or 1-2 shallow spiral grooves; with increase in the number of twists the nucleus stained more and more densely, became thinner and often looked like a small closely coiled spring; the densely staining, slender, rod-shaped nuclei showed either a faint twist of steeper pitch than the close spirals or none at all; the pale staining, long, rod-shaped nuclei showed no twist in general. Hemotoxylin and also the pyridin-silver method gave the best nuclear pictures.

The muscle fiber itself also exhibited definite spiral twisting. This spiralling could be either a right or a left twist; in a few instances a left twist was observed on one side of the nucleus while the opposite side of the muscle fiber showed a right twist. In the same field both right and left twists could be observed, though it appeared on counting as if one type usually predominated. Like the nuclei, not all the muscle fibers show a twist; in the same muscle ribbon or sheet, parts show the helicoid twisting which pass by insensible gradations to the non-twisted type. Usually when the muscle fiber showed a twist, a similar one with apparently the same pitch (ocular micrometer) was observable in the nucleus; not infrequently, however, a spiral muscle fiber is seen where the nucleus belonging to it shows no twist and is more or less vesicular. The best pictures were obtained with hematoxylin and eosin, iron hematoxylin, and Van Gieson stains.

The white fibrous tissue as demonstrated by Van Gieson's stain, shows beautiful helicoids, especially when the contracted spiral caecal fold of the rabbit is examined. Figure 1 gives a slightly schematic representation of what may be observed. These white fibrous cords or fiber aggregates run generally more or less at right angles to the course of the smooth muscle fibers in the caecal fold, the muscle fibers themselves being almost parallel to the long axis of this fold. When the plane of the section is

parallel to the long axis of the white fibrous tissue in the spiral caecal fold, these collagen fibers are seen to form helicoids which may have one type of twist throughout their course, or may exhibit one or several nodes of reversal where a right spiral changes to a left or vice versa (see Figure 1 at places indicated by arrows). A similar reversal of twist is seen when a series of old grapevine tendrils is examined.

In tangential sections of the rabbit's caecum showing all the layers, two sets of collagen fiber spirals may be seen running between the muscle fibers, one set parallel to the long axis of the muscle fibers, the other at right angles to this set; this arrangement is particularly noticeable at the base of the spiral fold.

The spiral twisting of the nucleus must express some of the nuclear fluids into the perinuclear spaces of the muscle cell, and the muscle cell subjected to a similar strain must in turn also filter out under this pressure some of its constituents into the fluids bathing the connective tissue fibers. It is readily conceivable that this process may alter the reaction of sarcoplasm as well of the tissue lymph. Thus a spiral contraction of the nucleus could initiate functional changes in the cell body including the sarcolemma of a smooth muscle, and a spiral contraction of smooth muscle similarly might affect the connective tissue elements surrounding it. It should be noted that a spiral twist beginning in the middle of a muscle cell, viz., at the nuclear site, must necessarily produce different twists above and below the nucleus: a right thread above and a left one below or vice versa, depending upon whether the twisting nuclear force was acting clockwise or counterclockwise. This is not the only way in which a reversal of twist may be secured. In a relatively rigid helicoid fixed at one end, rotation of the other end in the same direction as the existing twist will produce nodes of reversal, and a left helicoid can readily be changed into a right helicoid or vice versa. In a subsequent communication this will be described more fully.

Furthermore, it may be observed that these torsions can be utilized to explain certain morphological changes: for example, the change of an ovoid, vesicular pale-staining muscle nucleus into a slender rod-shaped, densely staining structure; or the change from a short thick muscle cell into a long narrow cell. Finally, the effect of torsion upon the length of the nucleus, cell body or connective tissue strand will depend upon the elasticity of the structure involved, which may perhaps be a quality fluc-

tuating with various conditions. If the structure is relatively inelastic, then its shortening must result if twisted into a helicoid; if more or less elastic, a lengthening may occur.

The helicoid twist described cannot be deprived of significance by classing it as an artifact or an abnormal state; if it is due to the reagents employed, the involved structures must have contained compounds different from those found in the non-twisted homologous structures and this means a different functional state. Abnormality on analysis simply means that a normal process has been increased or decreased beyond a certain level, for in no abnormal process is there ever the creation of a new function, there is merely the exaggeration in a positive or negative direction of capacities active or dormant in the cell.

2985

The time of ovulation in the menstrual cycle of the monkey, Macacus rhesus.*

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Available data for placing the time of ovulation in the sexual cycle of primates is meager in comparison to that for other mammals. Much of the evidence consists of the finding of early *corpora lutea* in the ovaries. Since in most cases it has not been possible to correlate the condition of the ovum discharged or its position in the tube with the stage of development of the corresponding *corpus luteum,* this evidence is incomplete.

Recently Corner[1] recovered an ovum from the tube of a monkey on the 14th day of the cycle. This was the first unfertilized tubal ovum of a primate to be recovered after being freed from the ovary. A degenerating ovum was also removed from the uterus on the 17th day of the cycle. In six other animals, all

* This work has been assisted by a grant from the Committee for Research on Sex Problems of the National Research Council.

1 Corner, George W., *Carnegie Inst. Cont. to Emb.*, 1923, xv, 73.

of which had menstruated within two weeks of the time of killing, no signs were found in the ovaries of recent or impending ovulation.

In the course of some experimental work upon injections of the ovarian follicular hormone into monkeys, operations have been performed at several intervals of the menstrual cycle. In the ovaries removed from a monkey on the first day of menstruation, there was no visible indication of either a large follicle or early *corpus luteum.*

A second monkey was operated on the 16th day of the cycle. One ovary was large and opalescent, although no individual large follicles could be seen from the surface. Some clear liquor was aspirated by means of a capillary pipette, but no ovum could be found. As this ovary was not removed, there was no way of determining whether the follicle was in a normal condition. A third monkey, operated on the 14th day, showed no signs visible on the surface of the ovaries of either a large follicle or recently formed *corpus luteum.* However, these two monkeys were young, having just attained sexual maturity.

A fourth monkey was operated on the 14th day after the appearance of the previous menses. A rupture point was visible on the surface of one ovary through which a hernia of luteal tissue protruded. The ampulla of the tube was distended to a semi-translucent state. The tube was removed, a hypodermic needle inserted into the uterine end and an ovum washed into a watch glass. On examination in warm Ringer's solution it was found to be completely surrounded by several layers of follicular cells. This may be interpreted as evidence of a very recent ovulation.

An operation was performed on a fifth monkey on the 10th day of the cycle. Conditions very similar to those in the fourth monkey were found, and a second unfertilized tubal ovum was recovered. These two ova are therefore the second and third unfertilized primate ova to be recovered from the uterine tubes after ovulation.

These ova were measured in warm Ringer's solution and fixed for further study in sections. A correlation of the degree of development of the corresponding *corpora lutea* is planned in the near future.

These results add two new instances to the relation of ovulation to the menstrual cycle in *Macacus rhesus.* One coincides with Corner's case of the 14th day, but the other shortens the period

at which ovulation may occur to the tenth day. On the basis of the recovery of these three unfertilized tubal ova, the time of ovulation in the menstrual cycle may be placed between the tenth and fourteenth days.

2986

The ovarian follicular hormone: a study of variation in pig, cow and human ovaries.

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It is now possible to measure quite accurately the amount of follicular hormone present in the ovaries of the larger mammals. The unit of measurement is the least amount of lipoid extract required to produce a definite physiological reaction in the spayed adult rat. This reaction to the injected hormone proves a good physiologic unit of measurement because it has a fairly sharp end point which may be determined accurately without sacrificing the test animal. It has been fully described and illustrated in earlier papers.[1] Briefly stated, the test consists of the induction of enough growth in the genital tract to cause the formation of a cornified layer in the vaginal epithelium. This growth requires about 48 hours and amounts to a complete replacement of the epithelial wall of the vagina. A positive test is indicated when cornified cells replace the leucocytes typical of the vaginal smear of the spayed control animal.

Material from pigs accurately timed with regard to its position in the 21 day oestrous cycle was collected by Dr. F. F. McKenzie, Department of Animal Husbandry, University of Missouri, in the course of his studies on reproduction.[2] The first ovary ex-

[1] (a) Allen, Edgar, and Doisy, E. A., *J. Am. Med. Assn.*, 1923, lxxxi, 819. (b) Allen, Edgar, Doisy, E. A., Francis, B. F., Robertson, L. L., Colgate, C. E., Johnston, C. G., Kountz, W. B., and Gibson, H. V., *Am. J. Anat.*, 1924, xxxiv, 133. (c) Doisy, E. A., Rolls, J. C., Allen, Edgar, and Johnston, C. G., *J. Biol. Chem.*, 1924, lxi, 711. (d) Allen, Edgar, and Doisy, E. A., *Am. J. Physiol.*, 1924, lxix, 577.

[2] McKenzie, Fred F., *Anat. Rec.*, 1924, xxvii, 185.

tracted was from a sow killed on the 19th day of the oestrous cycle, just as external signs of sexual activity were again appearing. This ovary weighed 7 grams and contained 9 large follicles of 10.5 mm. average diameter. The follicles were slit open and the whole ovary extracted with three changes of several times its volume of 95 per cent alcohol. The residue from evaporation of this extract was dissolved in corn oil. This solution, made up in a graded series of dilutions, was then tested by injection into spayed rats as outlined above. Results of these tests returned a total of 15 rat units of hormone.

The other ovary from this animal contained 8 large follicles of the same average size. Consequently the two ovaries were quite equally sharing function at this oestrous. The hormone content of the second ovary may therefore be estimated at about 13 rat units. This would make a total of 28 rat units for both ovaries of this sow on the first day of oestrus. It should be noted in this connection, however, that the production of 17 follicles at one oestrus in the pig, though not unusual, is more than the average number.

An ovary from another animal of this series was removed on the second day following the first appearance of external signs of oestrus. Ovulation had occurred and eleven ova were recovered from this one oviduct. There were eleven *corpora lutea* of the first or second day of development in this ovary. Tests of the extract were negative in doses up to an equivalent of one-half the total extract. The second ovary from this animal contained only one *corpus luteum,* consequently the ovary extracted was bearing practically all the function at this oestrous period. An estimation of the total hormone content of both ovaries would therefore be less than 2.1 rat units. Low yields have also been obtained from pig ovaries removed as late as one week before oestrus, but further tests must be made of these stages.

These results indicate a variation in the follicular hormone content from more than 28.0 rat units shortly before ovulation to less than 2.1 units shortly after ovulation in the ovaries of these two sows.

The cow material extracted was collected by Drs. H. S. Murphey and G. W. McNutt, Department of Veterinary Anatomy, Iowa State University. It was not possible to place the ovaries in accurate time relations in the cycle by observations on the animals before killing. The ovaries were grouped, however, by

correlating the condition of the genital tract and the visible ovarian structures with earlier results from observations upon normal animals.[3] These groups represented five different intervals of the 21 day oestrous cycle: (1) "in oestrus," (2) 1 to 5 days, (3) 5 to 10 days, (4) 10 to 17 days, and (5) 17 to 20 days after oestrus. Each group consisted of from 20 to 30 ovaries, both ovaries from each cow being included. Before the second extraction the ovaries were cut into thin sections and the follicles and *corpora lutea* counted and measured.

The extract of the group of 26 ovaries taken at oestrus, when the follicles were largest, returned a total of 60 rat units of hormone, or an average of 4.6 units per cow. It is considered that this represents the average minimum present in one large follicle 1 or 2 days before ovulation, for except in cases of dizygotic twins the cow usually ovulates only one ovum at each oestrus.

Extracts of the other four groups of cow ovaries each returned negative results in portions as large as one-third the total extract of that group. This means the presence of only very small amounts of follicular hormone in the ovaries of the cow at times other than oestrus. It should be noted in this connection that each of these four groups of cow ovaries contained from 8 to 11 large *corpora lutea* covering time intervals up to 21 days of development. It would seem, therefore, that the *corpus luteum* of the cow, like that of the pig, contains very little if any of the follicular hormone.

For purposes of comparison with these results reference is made to work done in collaboration with Drs. J. P. Pratt and E. A. Doisy in measuring the amount of follicular hormone in human ovarian tissues.[4] Briefly summarized: (1) The highest yield of hormone from human liquor folliculi was 7 rat units per cc. (2) The highest yield from human corpora was obtained from those removed at operation during the third week following the first day of the previous menstruation. This amounted to 3.7 rat units per cc. of luteal tissue (the volume of the corpora being measured by the amount of fluid displaced by the tissue after the first extraction. (3) Corpora removed during the fourth week of the menstrual cycle yielded 2 rat units per cc.

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[3] (a) Murphey, H. S., *J. A. V. M. A.*, 1924, viii (n. s.), 598. (b) McNutt, G. W., *J. A. V. M. A.*, 1924, xviii (n. s.), 556.

[4] Allen, Edgar, Pratt, J. P., and Doisy, E. A., *J. Am. Med. Assn.*, 1925, xcv, 399.

A summary of our analyses of ovarian tissues of these three mammals brings out several main points. (1) In the ovaries of cows and pigs there is a wide variation in the amount of follicular hormone present at different times in the cycle, a variation directly correlated with the size of the follicles. (2) The maximum attained during oestrus drops rapidly at about the time of ovulation and the newly forming corpora contain very little of this active substance. (3) Analyses of human ovarian tissues, besides showing a high hormone content in liquor folliculi, show also a considerable amount of the follicular hormone in the *corpus luteum.*

It has been assumed by most workers in this field that the endocrine function of mammalian ovaries is the same regardless of the species. There seem to be few differences in the formation of follicles and maturation of ova that would suggest otherwise. Anatomical differences have been described in the transition of follicles to *corpora lutea* in human beings and other mammals, but no functional discrimination has as yet been made among corpora which is typical of any group or species. The difference shown in our results may prove significant in explaining the differences in sexual phenomena in primates and in the lower mammals.

Review of the recent literature[5] discloses several differences between the menstrual cycle of primates and the oestrous cycle of pigs and cows. (1) The evidence for primates indicates that ovulation occurs in the intermenstrum. In the lower mammals it occurs toward the end of eostrus. (2) The oestrous cycle in the lower mammals is characterized by a short period at about the time of ovulation to which mating instincts are normally restricted, while in the primates studied the mating instincts may be diffused over practically all of the menstrual cycle. (3) The duration of the cycle in primates is a week longer than in the pig and cow. (4) The catabolic phase in the genital tract, whether it be hemorrhage or leucocytic infiltration, more closely follows ovulation in the oestrus than in the menstrual cycle.

The ovarian follicular hormone exerts a growth inducing influence upon the genital tract which raises it to maximum function including secretory activity of the glandular epithelium. It

[5] (a) Marshall, F. H. A., The Physiology of Reproduction, London, 1922; *Physiol. Rev.*, 1923, ii, 355. (b) Corner, G. W., *Physiol. Rev.*, 1923, iii, 457. (c) Murphey, H. A., McNutt, G. W., Zupp, B. A., and Aitken, W. A., *J. A. V. M. A.*, 1925, lxvii, 1.

also bears a causal relationship to mating instincts.[1b] Consequently the rapid increase in the reserve supply of this hormone in the ovaries of pigs and cows to a maximum at oestrus would result in a rise of the growth curve of the genital tract and also in a climax of mating instincts. The rapid drop following ovulation would remove this stimulus and allow the early succession of the degenerative phase. In the primates on the other hand, the high concentration of this hormone in the follicle, probably rising to a maximum during the latter part of the second week, and continuing in relatively high concentration in the *corpus luteum,* would extend considerably the period of influence of this hormone. This might account in part for: (1) the longer duration of the menstrual cycle, (2) the longer portion of the cycle during which mating instincts may be in evidence, and (3) the continuance of the anabolic and secretory phase for a considerable period after ovulation.

Peking Branch.

Peking Union Medical College, China, November 27, 1925.

2987

The effect of an exclusive milk diet on intestinal amoebae.*

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While working with culture rats, Kessel[1] noted that amœba-positive rats, given an exclusive milk diet for two weeks, were found to be amœba-negative when autopsied at the end of that time. It has been shown by Rettger and Cheplin[2] and Cannon and McNease[3] that a milk diet changes the flora of rats from a predominant *Bacillus coli* type to a predominant *Bacillus acidophilus* type. Hegner[4] further found that an exclusive milk diet is disadvantageous for the growth of Giardia, Trichomonas and Hexamitus in rats, the flagellates being reduced in numbers during the feeding. These facts together with the knowledge that milk is often prescribed as a beneficial diet during treatment for dysentery or for intestinal protozoan infections[5] led to the following experimental work on monkeys and on children to ascertain the possible value of a milk diet as a therapeutic measure in cases of infection with intestinal amœbae.

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* Contribution No. 69.

[1] Kessel, J. F., *Univ. Calif. Publ. Zool.*, 1924, xx, 489.

[2] Rettger, L. F., and Cheplin, H. A., The intestinal flora with special reference to the implantation of *Bacillus acidophilus*. New Haven, Yale Univ. Press, 1921, p. 135.

[3] Cannon, P. R., and McNease, B. W., *J. Inf. Dis.*, 1923, xxxii, 175.

[4] Hegner, R. W., *Am. J. Hyg.*, 1923, iii, 180.

[5] Routine Division Circular No. 16 of the South African Institute for Medical Research

In the work here recorded, the routine methods of examining stools, first by the iodine-eosin smear method and later by the iron-hematoxylin permanent smear method, were employed, similar to those established in the Parasitological Diagnostic Laboratory in Peking Union Medical College (Kessel[6]). Gram stains also were made, (1) prior to the milk feeding, (2) during the milk feeding and (3) after the conclusion of the experiment. These stains corroborated the fact that there is a change in the flora from a predominant Gram-negative type before feeding to a predominant Gram-positive type during the milk feeding.

Preliminary fecal examination was made of the monkeys and of the children on six or more days prior to starting the milk diet. The stools were then examined daily during the milk feeding and in the follow-up examinations two weeks after the last day of feeding and later two and three months after the last feeding. Six or more daily examinations were made at each follow-up period.

Two monkeys and two children positive for amœbic infection were kept in each experiment as controls and in each instance amœbae appeared in the stools throughout the period of the experiment.

The accompanying tables (I-III) giving the results of the experimental work, bring out the following points:

I. Boiled milk fed to monkeys for two weeks.

In a preliminary feeding experiment in which unboiled milk was used, two monkeys died of an infection apparently acquired in the milk. Hence boiled whole milk was fed during this experiment purely for precautionary measures. Although there was a reduction and in some cases a disappearence of the amœbae from the stools during the milk feeding, the amœbae, for the most part, were found again in the stools at the end of the first follow-up examination two weeks after the milk diet had been completed.

II. Unboiled milk fed to monkeys for two weeks.

Unboiled milk was again tried in this experiment, this time with no detrimental results to the health of the monkeys. It is to be noted that the results of this experiment are more satisfactory from a therapeutic point of view than those of Experiment I, since in this experiment 60 per cent of the monkeys became

[6] Kessel, J. F., *China Med. J.*, 1925., xxxxix, 85.

Tables showing results of exclusive milk diet.

TABLE I. Boiled Milk to Monkeys.

	No. 1			No. 2			No. 3			No. 4			No. 5		
	B. T.	D. T.	A. T.	B. T.	D. T.	A. T.	B. T.	D. T.	A. T.	B. T.	D. T.	A. T.	B. T.	D. T.	A. T.
E. dysenteriae	+	0	0	+	3	+	+	0	+	+	0	+	+	0	+
E. coli	0	0	0	+	0	+	0	0	0	+	0	+	+	0	+
Endolimax	+	4	+	+	1	+	+	1	+	+	4	+	0	3	+
Iodamœba	0	0	0	+	0	+	0	0	0	+	0	+	+	0	+

TABLE II. Unboiled Milk to Monkeys.

	No. 6			No. 7			No. 8			No. 9			No. 10		
	B. T.	D. T.	A. T.	B. T.	D. T.	A. T.	B. T.	D. T.	A. T.	B. T.	D. T.	A. T.	B. T.	D. T.	A. T.
E. dysenteriae	+	0	0	+	0	+	+	0	0	+	0	0	+	0	+
E. coli	+	0	0	+	0	0	0	0	0	+	0	0	+	0	0
Endolimax	+	0	0	+	2	+	+	0	0	+	2	+	+	0	+
Iodamœba	+	0	0	0	0	0	+	0	0	+	0	0	+	0	+
Chilomastix	+	0	+	+	2	+	+	+	+	0	0	0	0	0	0

TABLE III. Unboiled Milk to Children.

	No. 1—Age 10			No. 2—Age 10			No. 3—Age 9		
	B. T.	D. T.	A. T.	B. T.	D. T.	A. T.	B. T.	D. T.	A. T.
E. dysenteriae	+	0	+	+	0	0	+	0	0
E. coli	+	3	+	+	0	0	+	0	+
Endolimax	+	2	0	+	0	0	+	1	+
Iodamœba	+	0	0	+	0	0	+	0	+
Trichomonas	0	0	0	0	0	0	+	3	+
Chilomastix	0	0	0	+	0	+	0	0	0
Giardia	+	3	+	0	0	0	0	0	0

Explanation of Tables (I-III).

B. T.—Infection with protozoa determined by six or more daily examinations of feces, prior to milk diet.

D. T.—Number of times positive by daily examination for protozoa during milk treatment after the third day of feeding.

A. T.—Results of follow-up examinations after treatment.

permanently negative for *E. dysenteriae,* 100 per cent for *E. coli,* 40 per cent for *Endolimax,* and 80 per cent for *Iodamoeba.*

III. Unboiled milk fed to children for ten days.

Since the results obtained from giving a whole unboiled milk diet to monkeys were found to be promising, three children who were heavily positive for amœbae, but who demonstrated no important clinical symptoms at the time of the experiment, were placed on an exclusive unboiled milk diet for a period of ten consecutive days. Since the children objected to such a diet and began losing weight it was impossible to continue the milk diet longer. The results, however, show a marked decrease in the presence of amœbae during the feeding, and in Child No. 2 a complete and permanent riddance of the amœbic infection. Child No. 1 was cleared of *Endolimax nana* only and Child No. 3 of *E. dysenteriae* only.

From Tables II and III it will be seen that the flagellates did not respond so readily to the milk diet as did the amœbae.

CONCLUSIONS.

During the feeding of an exclusive raw milk diet there is almost always a reduction in the number of intestinal amœbae and in certain instances the intestinal tract has been cleared of *E. dysenteriae* and other forms and has remained clear for a period of three months following the conclusion of the experiment. This diet, however, cannot be recommended as a therapeutic measure since the results are so uncertain, the diet inadequate and offensive to some and since the possible dangers of infection in feeding raw milk are considerable.

2988

Susceptibility of field, house and laboratory rodents to infection with Leishmania donovani.

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After it has been demonstrated that the striped hamster *(Cricetulus griseus)* is easily infected with *Leishmania donovani*[1, 2] we desired to know how its susceptibility compares with that of the laboratory rodents previously used in the study of kala-azar. When the hypothesis of a rodent reservoir of kala-azar suggested itself, a study of the susceptibilities of the field and house rodents of an endemic area was indicated.

Rabbits and guinea pigs are known to be refractory to infection with *Leishmania.* White mice usually have been used; white rats less frequently and with less success. It was decided to study these two animals in order to compare them with the wild rodents. The forms investigated thus far are: (1) the giant hamster *(Cricetulus triton)*, (2) a vole *(Microtus* sp.*)*, (3) the Chinese house mouse *(Mus wagneri)*, (4) the house rat *(Mus rattus)*, (5) the white mouse *(Mus musculus albinus)*, a strain imported from America, and (6) the white rat *(Mus norvegicus (vel decumanus) albinus)*. The results of the study of this lot of white rats have been reported[3] but are repeated here for comparison with those of other rodents. So far as known, only the white rat and white mouse have been studied previously and there is lack of unanimity as to the susceptibility of the first of these.

The purpose of the present investigations was to determine whether the rodents named are susceptible to infection with *Leishmania donovani* and if so to what degree, and whether there is any tendency toward spontaneous recovery. Should such be

* Assisted by grants from the China Medical Board of the Rockefeller Foundation.

[1] Smyly, H. J., and Young, C. W., PROC. SOC. EXP. BIOL. AND MED., 1923-24, xxi, 354.

[2] Young, C. W., Smyly, H. J., and Brown, C., PROC. SOC. EXP. BIOL. AND MED., 1923-24, xxi, 357.

[3] Young, C. W., and Smyly, H. J. (in press).

found it was desirable to ascertain when recovery began and when it was complete.

Method. We have found that with our five laboratory strains of *L. donovani,* the liver and spleen from one positive hamster may be injected intraperitoneally into twenty new animals and that this dose will produce moderate to heavy positives in from one to two months. Doses for the rodents studied were made in proportion to the average weight of the species as compared with that of the striped hamster. Lots of a given species were inoculated at one time in order that each might receive the same number of organisms. This was necessary, because as yet there is no method of counting the non-flagellate form of *Leishmania.* It was planned to liver-puncture each animal ten days after inoculation in order to test whether it actually had become infected. This procedure had to be omitted in the case of some of the smaller species on account of the high mortality that resulted from the procedure. At ten-day intervals, estimated from the date of inoculation, two or three of the animals were killed with ether and autopsied aseptically. Smears and cultures (NNN medium) were made from the spleen, liver, bone marrow and heart blood. In some series, the peritoneal cavity was washed out with sterile Locke's solution and the washings centrifugalized. Smears and cultures were made from the sediment. Animals often died either as a result of liver puncture or from some intercurrent cause. Such carcasses were autopsied but as the organs became infected with bacteria soon after death, routine cultures were not made. In estimating the intensity of infection the following scale was used: + indicates less than an average of one *Leishmania* organism to each oil-immersion field (2.0 mm. objective; B and L 10X ocular); ++, between one and ten parasites; +++ between 10 and 100; ++++, between 100 and 500. The organisms were estimated approximately; not counted. Degree of susceptibility was taken to be indicated by the number of parasites per field in the smears. If none of a given species showed more than ++ or +++ we have said that such animals were only moderately susceptible to infection with *Leishmania donovani.* The criterion of "a tendency toward spontaneous recovery" was considered to be a decrease in the intensity of infection in the later members of a series.

Results. Elsewhere[2, 3] it has been shown that striped hamsters begin to show positive liver punctures before the tenth day after

inoculation, and that with rare exceptions there is no tendency toward spontaneous recovery.

Giant Hamster (two series of 19 and 15 hamsters respectively). Of 34 giant hamsters inoculated, 26 were positive at autopsy, four were negative and the organs of four were decomposed and therefore unreliable for diagnosis. The animals died or were killed at intervals of 25-320 days after inoculation. No tendency toward spontaneous recovery was noted. The organs of these animals frequently showed ++++ infections so that we consider them nearly or quite as susceptible as striped hamsters.

Vole (one series of 10 voles). On account of the difficulty in obtaining sufficient numbers of these animals and of keeping them alive in captivity, only one small series was studied. They showed infections of moderate intensity (++ or +++) after 13-21 days. No tendency toward recovery was seen in the individuals dying 37 and 38 days after inoculation.

House Rat (one series of 9 rats). This rodent is also difficult to maintain in captivity. None of the animals in the single series studied became heavily infected. Only one showed +++ in the spleen. Both rats that died 46 days after inoculation showed light (+) infections of the spleen and liver.

House Mouse (four series of 9, 17, 19, and 20 mice respectively). On the other hand, house mice are easily maintained and those dying 100-140 days after inoculation were moderately (++) to heavily (++++) positive. If there was any tendency toward spontaneous recovery, it was not marked.

White Rat (one series of 10 rats). In the one series studied, there were no infections above +, the smears became negative after 30 days and the cultures (except from the peritoneal cavity) also with the exception of that from the spleen of one 37 day animal.

White Mouse (two series of 15 and 18 mice respectively). These white mice became moderately infected (++, +++; rarely ++++) after about forty days. Animals killed after 70 days showed the same intensity. Two killed after 82 days were only + (one spleen smear ++) but no definite conclusion regarding tendency toward spontaneous recovery can be drawn from the small number of animals examined..

Discussion. None of the series of animals in which some members showed an apparent tendency to recover, was sufficiently prolonged to demonstrate undoubted spontaneous recovery. Such a tendency seemed to be present in house rats, white rats and to a less extent in white mice but the numbers of these rodents killed at each interval were too few to make the findings unequivocal. All the field rodents studied, as well as the house mice remained heavily infected throughout the period of observation

Conclusions. 1. The giant hamster *(Cricetulus triton)*, a vole *(Microtus* sp.) and the Chinese house mouse *(Mus wagneri)* are markedly susceptible to infection with *Leishmania donovani.* These showed no tendency toward spontaneous recovery during the periods covered by the present studies.

2. The house rat *(Mus rattus)* white rat *(Mus novegicus (vel decumanus) albinus)* and white mouse *(Mus musculus albinus)* show infections of only moderate intensity and seem to exhibit a tendency to recover spontaneously. However none of the series was long enough to show undoubted and complete recovery.

2989

A search for field and house rodents naturally infected with kala-azar.

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The striped hamster *(Cricetulus griseus)* has been shown to be very susceptible to infection with kala-azar.[1] Its southern range corresponds, so far as it has been studied, with the distribution of kala-azar in China. These two facts together with other obser-

* Assisted by grants from the China Medical Board of the Rockefeller Foundation.

1 Smyly, H. J., and Young, C. W., PROC. SOC. EXP. BIOL. AND MED., 1923-24, xxi, 357.

2 Young, C. W., Smyly, H. J., and Brown, C., PROC. SOC. EXP. BIOL. AND MED., 1923-24, xxi, 357.

vations on the epidemiology of the disease, suggested the possibility that this hamster or some other field rodent may be a reservoir of *Leishmania donovani*. Assuming such a source, man might be infected in his dwelling directly from the animal or indirectly by means of some domestic rodent (rat or mouse) which had in turn become infected from an invading field rodent. In either case the probable vector or intermediate host would be some rodent ectoparasite. With this rodent-ectoparasite hypothesis in mind we have: (1) searched for hamsters and other field and house rodents naturally infected with kala-azar; (2) tested for susceptibility to infection with *Leishmania donovani* the common field and house rodents from an endemic area; and (3) studied the natural ectoparasites of these rodents and their capabilities as transmitters of the disease. The present paper is a brief statement of the results of the first of these investigations. The animals recorded comprise (1) those killed and examined primarily for the purpose of searching for naturally infected animals; (2) those that died in the laboratory before they had been submitted to any experimental procedure and (3) such stock rodents as were accidentally killed by liver puncture. From this study were excluded (1) all rodents that had received any injection or had been submitted to any manipulation other than liver punture; (2) all carcasses that showed decomposition of the organs; and (3) all animals regarding whose exact origin and subsequent history there was any doubt. Examination consisted in making smears of at least the spleen and liver, staining with Wilson's stain and studying microscopically.

Field Rodents. More than 3500 striped hamsters were sacrificed in the direct search for natural infections. In less than fifty of these, smears were not examined but the spleen and liver were injected intraperitoneally into tested negative hamsters. Later these inoculated animals were killed and smears for their organs were examined microscopically. Enlargement of the spleen resembling more or less that found in early or moderately advanced kala-azar, was occasionally encountered. The cause of this enlargement was not determined. Trypanosomes were seen in some of the rats but in none of the other rodents here recorded.

Of the 4480 striped hamsters included in this study only one was found to be infected with *Leishmania donovani*.

H-8056 was brought to the Kala-Azar Field Studies Laboratory from Lo T'o Shan between October 1st and 15th, 1924. It

was placed with nine others of the same lot in a cage. On November 29th the ten hamsters were killed with ether and autopsied. The smears from this hamster showed *Leishmania donovani* in large numbers (spleen +++, liver +++, marrow ++++, heart blood +). The spleen was ground and suspended in sterile Locke's solution and injected intraperitoneally into a tested hamster (No. H-7798). The liver was similarly inoculated into ten tested negative hamsters (Nos. H-7799-7808). Of these eleven inoculated animals, eight were found to be infected when autopsied later and three were negative.

More than 3500 of the striped hamsters examined were captured during the months of October and November, 1924. The dates of taking the others were scattered throughout the rest of the year.

Four hundred giant hamsters *(Cricetulus triton)* and 134 voles *(Microtus* sp.) were examined. All were negative.

House Rodents. The house rodents studied were the black rat *(Mus rattus)* and the Chinese house mouse *(Mus wagneri)*. These were divided into those caught in "clean" houses, *i. e.,* buildings not occupied by kala-azar patients; and those taken in "kala-azar" houses, *i. e.,* buildings that were the homes of persons suffering from that disease. All the 400 rats from "clean" houses and the 210 from "kala-azar" houses were negative, as were the 114 mice from "clean" and 57 from "kala-azar" houses.

Discussion. Of the 5819 rodents examined, one, a striped hamster, was found to be infected with kala-azar. There is no reason to doubt that this is an authentic instance of natural infection. On the other hand it should be pointed out that infected hamsters were kept in the same building though in a separate room. Moreover, the animal was killed six to eight weeks after it was brought from the field. The fact that only 0.02 per cent of the striped hamsters were positive while about 5 per cent of the inhabitants of the nearby villages of Camel Mountain (Lo T'o Shan) and Lion Mountain (Shih Tzu Shan) have the disease indicates that these rodents are not the source of human kala-azar. Even if this animal was naturally infected, it does not follow that *Cricetulus griseus* is the reservoir of kala-azar. During the winter's trapping no field rodents were caught in village houses though a few house mice were taken in the fields throughout the year.

The number of house rodents examined was not large enough to form a basis for definite conclusions, but the evidence so far as it goes is against domestic rodents being concerned with the spread of kala-azar.

2990

Attempts to transmit kala azar by means of rodent lice, haematopinus sp.

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On account of the susceptibility of the Chinese hamster to infection with kala-azar we have considered in another paper[1] the possibility of these and other rodents being reservoirs of the disease, the transmission in such case being effected by means of their ectoparasites from the rodents to other rodents and man. In this connection a number of rodent ectoparasites have been studied with regard to their capabilities as transmitters of kala-azar. The rodents examined have been chiefly the striped hamster *(Cricetulus griseus)*, the giant hamster *(Cricetulus triton)*, a vole *(Microtus* sp.*)*, the Asiatic house mouse *(Mus wagneri)* and the house rat *(Mus rattus)*. Their ectoparasites have been limited chiefly to fleas, bloodsucking mites *(Gamasidae)*, ticks, non-bloodsucking mites *(Myobia)* and lice (Pediculidae, *Haematopinus* spp.). The fleas, Gamasid mites and ticks have been found only on the house rodents, while the lice have been found on both field and house rodents, each having its own species of louse. The present paper is a summary of the studies made on the respective species of *Haematopinus* of the two hamsters.

In addition to the possible rôle of lice in connection with the rodent-ectoparasite hypothesis, a further reason for studying these insects lay in the fact that our stock animals tended to more

* Assisted by grants from the China Medical Board of the Rockefeller Foundation.

[1] Young, C. W., and Hertig, M., PROC. SOC. EXP. BIOL. AND MED., 1925-6, xxiii, 395.

or less lousiness. No practicable method was discovered of ridding the hamster completely of their parasites. Dipping in warm two per cent Izal solution was fairly effective with negative hamsters, but the inoculated animals seemed unable to withstand the inevitable chilling. It was thus important to discover whether it was possible to disregard the presence of lice in our other studies.

We have endeavored to determine (1) whether *Leishmania donovani* in any stage may be demonstrated in lice from positive hamsters, and (2) whether lice can transmit kala-azar.

I. *Occurrence of Leishmania in lice from positive hamsters.* Leishman-Donovan bodies are frequently demonstrable in smears of peripheral blood of human patients, and Young[2] has shown by cultural methods that the organisms are usually present in the peripheral blood. This point has not been determined directly for the hamsters, although heart-blood smears from heavily positive hamsters at autopsy are usually positive for Leishmania. However, in another study to be reported later, we have shown that sandflies *(Phlebotomus* sp.*)* fed on both striped and giant hamsters may develop flagellates of Leishmania in their intestines (thus confirming for Chinese sandflies the general phenomenon of exflagellation of Leishmania as described by Knowles, Napier and Smith.[3]) These parasites are thus shown to be present at least at times in the peripheral blood of both hamsters. Lice constantly present on the hamsters and feeding frequently would seem to have an opportunity for ingesting Leishmania.

Microscopic examination. Smears from many lice from heavily positive hamsters have been made, but in no case has even one Leishmania been found. A total of 153 striped-hamster lice and 329 giant-hamster lice have been thus examined and found negative.

Inoculation Experiments. Lice from positive hamsters were teased up in Locke's solution and injected intraperitoneally into tested negative striped hamsters. The lice were usually teased up individually in small drops of water on a slide, most of this suspension then being transferred to a larger volume for injection. Sufficient cellular debris remained behind in the little indi-

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[2] Young, C. W., and Van Sant, H., *J. Exp. Med.*, 1923, xxxviii, 233.

[3] Knowles, R., Napier, L. E., and Smith, R. O. A., *Indian Med. Gaz.*, 1924, lix, 593.

vidual drops to serve as control smears for the actual lice injected. The results are summarized as follows:

Striped-Hamster Lice. Twelve lots of lice, taken usually after death, from seven heavily positive striped hamsters were injected into 13 tested negatives. The lots consisted of 7-35 lice each, of all stages from young nymph to adult, and the period from removal to injection was 1 to 26 hours, the lice being kept at room temperature or in an incubator at 22° C.

Results. Positive results were secured in three instances as follows:

(a) Two tested negative hamsters were injected with a suspension of one lot of 35 lice about two hours after removal from carcass. At death after 143 and 193 days respectively autopsies showed both hamsters to be heavily positive. Smears of 35 other lice taken at the same time from the same hamster failed to reveal any Leishmania.

(b) A lot of 15 lice held at 22° C. 24 to 26 hours, were dead but the tissues were still fresh. A tested negative hamster inoculated with these was found to be positive +++ when sacrificed after 265 days. Another hamster was inoculated with a suspension of 21 living lice of the same lot, but since it died after only 32 days the negative findings at autopsy are inconclusive. Smears from the lice actually injected were negative.

(c) A tested negative hamster injected with a lot of 25 lice held 3 to 6 hours, on being sacrificed after 211 days was positive ++++. Control smears of the lice injected were negative.

The other eight hamsters were all negative at autopsy after 28, 34, 138, 206, 261, 297, 297 and 298 days respectively.

Giant-Hamster Lice. Nineteen lots of lice from nine positive giant hamsters were injected into 22 tested negative striped hamsters. The lots consisted of 6 to 42 lice each (average 21) of all stages, and were held at room temperature or at 22° C., six hours or less in five cases, 20 to 30 hours in nine cases and 48 to 72 hours in the remaining four. Control smears, made in all but two cases, were negative.

Results. Three hamsters, still living, were negative on liver puncture after 71, 71 and 78 days respectively. Autopsies of the remaining 19 hamsters were completely negative after 36, 63, 157

and 192 days respectively in five cases, and after 216 to 279 days in the other 14 cases.

Discussion. The failure to find Leishmania in smears of lice from positive hamsters indicates that there is no development of the parasites in the louse, but the positive inoculation results show that at least in the case of the striped-hamster louse the organisms may be present in such small numbers as to escape detection in smears, but nevertheless be capable after two to 26 hours of giving rise to infection when injected into negative hamsters.

II. *Attempts to transmit kala-azar by means of hamster lice.* The method used was to bring into close contact with one another a tested positive and a tested negative hamster, which were naturally lousy, or to which lice were added. Each pair of hamsters was kept in a porous earthen pot with fine sand in the bottom. Muslin tied over the top, with vaseline smeared liberally around the edge of the pot, prevented insects from either entering or escaping. Unfortunately the hamsters could not be left free within the pot because of their cannibalistic habits in captivity. Each pair was confined in a cubical, woven-wire cage, 10 cm. on each side, the animals being separated from one another by a central, vertical partition made of wire window screen. The sand and porous walls absorb any excessive moisture and the accumulation of dry feces and remains of the beans given for food, seem to have no ill effects. The extent to which the lice migrated from one hamster to the other was not determined. That the lice left the hamsters at times was shown by the fact that they were frequently to be found on the sand under the cage, and that in those cases where lice were at first present on only one hamster, they could soon be demonstrated on the other. At any rate, the opportunities for exchange of lice were excellent since in spite of the screen partition the hamsters were kept virtually in contact with one another.

Striped hamster series. A total of 15 tested negatives were kept in pots with lousy positive hamsters until they died or were sacrificed, the periods of time being 24, 30, 78, 90, 145, 158, 210, 233, 235, 264, 276, 286, 295, 308, and 357 days respectively. All were negative at autopsy.

Two tested striped hamsters received each about 75 lice taken from a heavily positive carcass, and were kept separately in earthen pots, there being no exposure to positive hamsters. The

two were sacrificed after 271 days of such isolation and were both found to be negative.

Giant hamster series. A total of ten tested negative giant hamsters were kept in pots with lousy positive giant hamsters. In two cases the animals died and carcasses were decomposed. The other eight at autopsy after 22, 23, 52, 63, 116, 125, 231 and 286 days were found to be negative. The giant hamsters withstood the conditions of the pots much less successfully than the striped hamsters due in large part to their extreme lousiness, which hastened their death.

A tested negative giant hamster received 15 to 20 giant-hamster lice, a few of which had fed on a heavily positive striped hamster. This giant hamster was kept by itself in a pot and at autopsy after 229 days was found to be negative.

Discussion. It is thus seen that there was no demonstrable transmission of kala-azar from lousy positive to negative hamsters kept in close proximity over relatively long periods of time.

Summary. (1) No development of *Leishmania donovani* in the two species of hamster lice studied was demonstrated, but these organisms occasionally survive for a short period in the striped-hamster louse.

(2) Attempts to transmit kala-azar from hamster to hamster by means of hamster lice were completely unsuccessful.

2991

Attempts to transmit kala azar by means of bedbugs (cimex sp.).

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Since Patton in 1907 demonstrated that *Leishmania donovani* would develop into the flagellate stage and multiply in the intestine of the bedbug, a number of investigators have studied the bedbug as possible vector of both kala azar and oriental sore.

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(For critical review of the literature and bibliography see Wenyon, Tropical Diseases Bulletin, 1922, xix, 1-18.) Although the organisms of both kala azar and oriental sore are able to produce abundant growths of flagellates in both *Cimex lectularius* and *C. rotundatus,* every effort to transmit these diseases to experimental animals and even to man by means of the bites of bedbugs has failed, and attempts to produce leishmaniasis by inoculating such infected bugs into animals has failed with a single exception. Shortt and Swaminath[1] fed bedbugs on patients and then injected these into mice. Cultures of Leishmania were obtained from one of five mice after 123 days. Furthermore, flagellates have not been found in hundreds of bugs taken from the bedding of kala azar patients (Mackie,[3] Shortt and Swaminath[2]). In spite of the many failures to transmit, several investigators, notably Patton,[4] have maintained the bedbug to be the vector of kala azar.

Although there are grave objections to the bedbug as vector on epidemiological grounds, and the many uniformly negative transmission experiments fail to incriminate the bedbug as vector, we have thought it desirable to attempt to transmit kala azar, using the highly susceptible striped hamster. The lack of such suitable experimental animal has been a serious handicap to previous workers. The present paper is a report of transmission experiments made with *Cimex lectularius,* the common bedbug of North China, and with the closely related *Cimex pipistrelli* parasitic on bats. The work on the latter was undertaken along with the investigation of rodents and other animals and their ectoparasites as possible reservoirs and vectors of kala azar. The bat Cimex, living with the bats in the walls of houses, is frequently found in large numbers inside the house and occasionally feeds on man. One of us was bitten while collecting them. The investigation of the bat Cimex was especially desirable on account of its association with man and the ready development of Leishmania in other species of Cimex.

Material and Methods. Abundant *Cimex lectularius* material was obtained from various sources in Peking and Hsüchowfu, Kiangsu, not associated with kala azar patients or infected animals. Specimens of *Cimex pipistrelli* were found in small num-

[1] Shortt, H. E., and Swaminath, C. S., *Indian J. Med. Res.,* 1924, xi, 965.

[2] Shortt, H. E., and Swaminath, C. S., *Indian J. Med. Res.,* 1925, xiii, 143.

[3] Mackie, F. P., *Indian J. Med. Res.,* 1915, ii, 942.

[4] Patton, W. S., *Indian J. Med. Res.,* 1922, ix, 496.

bers on bats in Hsüchowfu, but most of our material was obtained from a certain room in a foreign house in Hsüchowfu, the outer wall of which harbored many bats. Of three adult bat bugs examined soon after being brought to the laboratory, all three were infected with trypanosomes or crithidiform flagellates. The few "wild" nymphs examined were negative for flagellates. No trypanosomes were found in the bats from which some of these bugs were taken.

The method followed was that used in experiments with hamster lice described in another paper.[5] A pair of striped hamsters consisting of a tested positive and a tested negative were kept in a partitioned cage in an earthen pot covered with muslin and made insect-proof by means of a vaseline seal between the muslin and the edge of the pot. Both species of Cimex thrive in such pots. They feed readily on the hamsters and use as breeding and hiding places either pieces of corrugated cardboard wired to the cage, or the dry bean hulls in the bottom of the pot.

TRANSMISSION EXPERIMENTS.

Cimex lectularius Series. A total of eleven tested negative striped hamsters have been kept in pots with positives and bedbugs. Of these six at autopsy after 112, 166, 261, 286, 286 and 286 days respectively were negative. The remaining five are still in the experimental pots, but were all negative on liver puncture after 176, 183, 194, 194 and 194 days respectively of contact with positive hamsters and bedbugs.

Cimex pipistrelli Series. A total of ten tested negative striped hamsters have been kept in the pots with tested positives and bat bugs. Four of these were autopsied after 89, 114, 161 and 162 days respectively and were found to be negative. The other six are still in the pots but were negative on liver puncture after 112, 112, 158, 158, 158 and 158 days respectively.

Discussion. In these two series of experiments, in the presence of abundant insects and with close contact of heavily positive and negative hamsters known to be highly susceptible to kala azar, no transmission has been demonstrated. This is in accord with the results of previous investigators. It may be noted that comparable "experiments" with man as subject are taking place

[5] Young, C. W., and Hertig, M., PROC. SOC. EXP. BIOL. AND MED., 1925-6, xxiii, 395.

constantly in most of the kala azar houses in China. The majority of kala azar cases in China are solitary, *i. e.*, there is only one infected person per household, with no obvious special relationship to other cases in the vicinity. Adults and children frequently sleep crowded together, the patient among them, and bedbugs are usually plentiful. If bedbugs were the vectors of the disease a succession of cases among the members of a kala azar household would be expected. Our observations indicate that this, in general, is not the case in China.

These transmission experiments are still in progress and will be extended. In addition the survival of the parasites in both species of Cimex is being studied. Bugs are fed on positive hamsters and after various intervals are injected into tested negative hamsters. In the few cases thus far in which the time since inoculation has made conclusive results possible, these have been entirely negative.

Summary. Attempts to transmit kala azar from heavily infected hamsters to highly susceptible negative hamsters by means of *Cimex lectularius* and *Cimex pipistrelli* have been unsuccessful.